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(71) Applicant *(for all designated States except US)*: CANCER RESEARCH CAMPAIGN TECHNOLOGY LIMITED [GB/GB]; Cambridge House, 6–10 Cambridge Terrace, Regent's Park, London NW1 4JL (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: GALLAGHER, John, Thomas [GB/GB]; 93 Racecourse Road, Wilmslow, Cheshire SK9 5LW (GB). PYE, David, Alexander [GB/GB]; 12 Buchanan Drive, Hindley Green, Wigan, Lancashire WN2 4HJ (GB).

(74) Agent: H.N. & W.S. SKERRETT; Charles House, 148/9 Great Charles Street, Birmingham B3 3HT (GB).

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(54) Title: HEPARIN–BINDING GROWTH FACTOR DERIVATIVES

(57) Abstract

Covalently cross–linked conjugates of heparin–binding growth factors and heparin or heparan sulphate oligosaccharides which can be used as therapeutic agents for modulating the biological activity of such growth factors and/or for targeted delivery of drugs are disclosed. Such conjugates enable exogenous growth factors to be administered to mammals for medical treatment so as either to promote or to inhibit growth factor biological activity, or to act as targeting carriers of drug molecules linked thereto.

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# HEPARIN-BINDING GROWTH FACTOR DERIVATIVES

## FIELD OF THE INVENTION

The present invention relates to the field of biochemistry and medicine. More particularly, it concerns certain novel bioactive derivatives of heparin-binding bioactive proteins or polypeptides present in biological systems, especially certain growth factors or cytokines such as fibroblast growth factors (FGF's) for example.

## BACKGROUND

In complex multicellular living organisms, for example humans and other mammals, it is well known that various aspects of cell development and differentiation, migration, growth and/or proliferation, involving in some cases cell-cell interactions, are often under the control of or are regulated by various extracellular mediators or cytokines, commonly referred to as growth factors, which are generally specialised soluble proteins or polypeptides secreted by cells of the tissues concerned.

In this specification, the term "growth factor" is used to denote generally all such bioactive extracellular control or regulatory proteins or polypeptides, in their native form or otherwise, which interact with external protein domains or binding sites of specific membrane-bound signal-transducing receptors of target cells and activate said receptors thereby to produce various effects in relation to cell development, including either stimulation or inhibition of cell division and control of cell differentiation. In some cases, different effects may be produced in different types of cells, or in the same cells under different conditions such that these growth factors act as development regulators.

Such growth factors, of which many have already been isolated and subsequently synthesised using recombinant DNA technology, are believed to

act through a variety of mechanisms that result in modulation of gene expression, but in general their effect seems to be mediated through the initial above-mentioned interaction with external protein domains or binding sites of specific membrane bound signal-transducing receptors of target cells, these receptors thereby being activated to bring about a chain or sequence of intracellular biochemical events.

Many of these growth factors have a characteristic high binding affinity for heparin or for the glycosaminoglycan (GAG) heparan sulphate (HS) which is the polysaccharide constituent of heparan sulphate proteoglycans (HSPG's) that are found on the surface of most animal cells and in the extracellular matrix (ECM). All such growth factors are referred to in the present specification as "heparin-binding growth factors". In at least many cases interaction with cell surface heparan sulphate appears to be an essential prerequisite for these growth factors to exercise their biological activity, the role of such heparan sulphate being to act as a co-receptor necessary for activation of the growth factor cell surface signal-transducing receptors or, in some instances, its role may be primarily to sequester and concentrate the growth factor molecules at the cell surface.

Among the heparin-binding growth factors that seem to require heparan sulphate in order to activate their cell-surface signal-transducing receptors of target cells are the members of the fibroblast growth factor (FGF) family, vascular endothelial growth factor (VEGF), hepatocyte growth factor (scatter factor) and heparin-binding epidermal growth factor (HB-EGF). Other heparin-binding growth factors include, for example, Interleukins (IL) 1α, 1β, 2, 3, 4, 6, 7, 8, 10 and 12, PDGF (platelet derived growth factor), TNFα (tumor necrosis factor), IGF I and II (insulin-like growth factor), IGFBP 3 and 5 (insulin-like growth factor binding protein), TGFβ (transforming growth factor), Interferon gamma (IFN-γ), Purpurin (retinal survival factor), Amphiregulin, Schwann cell mitogen, Pleiotrophin (p18), MSF (migration

stimulating factor), HBNF (heparin-binding neurite promoting factor), NEL-GF (neu/erb B2 ligand-growth factor), MK (midkine factor), Platelet Factor-4 (PF4), GM-CSF, MIP (macrophage inflammatory protein) 1 alpha and 1 beta, SDMF (smooth muscle cell derived migration factor), GDNF (glial cell line derived neural growth factors), MDC (macrophage derived chemokine), BMP-2, NAP-2 (neutrophil activating peptide), endostatin, angiostatin and lymphotactin.

Within the FGF family, of which there are presently nine known members, one growth factor which is especially important and which has been extensively studied is that known as basic fibroblast growth factor, bFGF or FGF-2. A second main form is acidic fibroblast growth factor, aFGF or FGF-1, which is similar to bFGF and has partial amino acid sequence identity but differing isoelectric points. FGF's are present in a wide variety of mammalian tissues; they appear to function in both normal and in diseased physiological states as important signalling molecules involved in regulation of cell growth and differentiation and they act as potent mitogens that stimulate proliferation in a range of cell types. A review by D Gospodarowicz of some of the characteristics and properties of FGF's is to be found in *Cell Biology Reviews* (1991) 25 (4), pp 305-314.

In particular, basic fibroblast growth factor (bFGF) appears to have an important role in processes such as embryonic development, wound repair and tumour growth due to its ability to stimulate angiogenesis, and it has been specifically implicated as being directly concerned in various disorders or degenerative conditions involving cell proliferation, including for example diabetic retinopathy, capsular opacification following cataract operations, restenosis after angioplasty, tumour angiogensis, and various forms of chronic inflammation including rheumatoid arthritis. It delivers its signal to cells by binding with specific cell surface tyrosine kinase receptors ($K_d$ 10-500 pM), such as receptors which are the expression products of the gene *flg*, that

generate intracellular signals. However, the mode of action of bFGF and similar growth factors or cytokines is complex and, as already indicated, it appears also to involve an interaction with the heparan sulphate component of heparan sulphate proteoglycans (HSPG's) ($K_d$ 5-50 nM) on the cell surface or in the extracellular matrix of mammalian cells. Experimental work has shown, for example, that in cells which are deficient in heparan sulphate (HS) synthesis the *flg* receptor will not respond to bFGF, but that addition of heparin or heparan sulphate (HS) can restore responsiveness, illustrating that in at least many cases such growth factors need to be activated by interaction with polysaccharides such as HS or heparin before they can exert their biological effect. Thus, although full details are not completely understood the concept has emerged of a dual-receptor mechanism, at least for the action of bFGF, and much experimental work has been carried out to try to locate and identify a supposed bFGF binding site in HS. HS is probably the most complex mammalian glycosaminoglycan (GAG), consisting of a linear polysaccharide chain having an ordered arrangement of domains rich in N- and O- sulphated groups, in which the basic disaccharide repeat unit consists of glucuronic acid or iduronic acid linked to an N-sulphated glucosamine, i.e. GlcA/IdoA-$GlcNSO_3$, spaced apart by extensive regions of low sulphation in which N-acetylated disaccharides (GlcA-GlcNAc) predominate. Since bFGF is a heparin-binding growth factor the sulphated domains which contain "heparin-like" regions would be expected to provide the most likely location of the bFGF binding site, but the size of these domains, their sulphation pattern and their iduronic acid content are highly variable, depending to some extent on the source of the heparan sulphate.

Using selective depolymerisation methods involving enzymes such as heparitinase which cleaves HS chains only in regions of low sulphation, together with various analytical techniques and affinity chromatography, heparin sulphate bFGF-binding oligosaccharides have been isolated and

sequenced, as described for example in WO 93/19096. It has been found that for significant binding of bFGF to occur, such oligosaccharides must generally contain at least five monosaccharide residues including a 2-O-sulphated iduronic acid, but mere capability of binding to bFGF is not necessarily in itself sufficient for such oligosaccharides to enable the growth factor to bind to and activate its cell surface receptors so as to exercise its biological activity. For this to occur, earlier experiments indicated that HS (or heparin) oligosaccharides must contain at least six disaccharide residues (degree of polymerisation, dp $\geq$ 12), and in contrast shorter length oligosaccharides that bind bFGF are capable of acting as inhibitors of the growth factor biological activity. These aspects, and the potential importance of the sulphation pattern in promoting biological activity, are further discussed to some extent in various published papers, e.g. G. C. Jayson and J. T. Gallagher (1997), "Heparin oligosaccharides: inhibitors of the biological activity of bFGF on Caco-2 cells", *Brit. Journal of Cancer* 75(1), pp 9-16, and D. Coltrini *et al*, (1994) *Biochem. J.* 303, pp 583-590. Later work, however, has indicated that in some cases even shorter HS or heparin oligosaccharides, e.g. containing five disaccharide residues (dp = 10), may in some cases have a stimulatory biological activity.

Although most studies have been carried out with bFGF, as already indicated above it is believed that aFGF and other members of the FGF family, as well as many other heparin-binding growth factors, including in particular VEGF and hepatocyte growth factor, have a similar requirement for heparan sulphate, or fragments thereof, in order to be able to interact with their cell surface receptors and exercise their biological activity. The mechanism is believed to be similar to that with bFGF except that somewhat different size restrictions may apply (e.g. for promoting stimulatory activity in respect of VEGF the HS oligosaccharides required are likely to be in the range dp8 to dp20) and different patterns of sulphation may be effective, as suggested by Guimond *et al* (1993), "Activating and Inhibitory Heparin Sequences for

FGF-2 (Basic-FGF)" *The Journal of Biological Chemistry*, 268, pp 23906-23914.

Whatever be the precise requirements and mechanism of these interactions, however, since these growth factors or cytokines such as FGF clearly have such an important and wide-ranging role in controlling or regulating cellular processes that are responsible both for maintaining or restoring a normal physiological state or for promoting certain disease states, the possibility of controlling or modulating their activity for the purpose of therapeutic treatment has been the subject of some attention. Thus, some consideration has already been given for example to the development and use of agents which would block the cell surface signal-transducing binding receptors, or which would act as antagonists or agonists to interfere with a necessary preliminary binding interaction between the growth factors and a cell surface proteoglycan or glycosaminoglycan, in order to inhibit growth factor activity.

Proposals for inhibiting the activity of growth factors such as bFGF in this manner have included the administration of short HS or heparin oligosaccharides which would act as competitive inhibitors, binding to sites on the growth factor and/or growth factor receptors without causing activation. It will be appreciated that inhibiting the activity of such growth factors can be especially relevant and important in connection with treatment of some cancers, bFGF for example being a potent mitogen and motility factor for a variety of cell types and being strongly implicated in tumour angiogenesis. Furthermore, bFGF and other members of the FGF family are produced ectopically by a number of human malignant tumours and cell lines derived from them, these including human stomach cancers, gliomas and breast carcinomas. Moreover, high-affinity receptors for bFGF are overexpressed in a number of tumours, such as human gastric and breast tumours. It is thus clear that regulation of bFGF activity could provide a target for therapeutic control of tumour growth.

In other situations, however, increased growth factor activity may be desired and beneficial for promoting healing or tissue repair, as for example in the case of wound healing and bone healing, leading to proposals for the clinical use and administration of exogeneous growth factors as therapeutic drugs and already some clinical trials in connection with bFGF have taken place. In the case of wound healing, the effective administration of bFGF should be especially useful in the treatment of conditions such as non-healing wounds and duodenal and venous ulcers. Moreover, by virtue of its angiogenic properties, this growth factor, and also VEGF, should also be useful in providing a therapeutic agent for reducing damage after a coronary block or for targeting blood vessels and maintaining the integrity of the endothelium so as to prevent thrombosis, e.g. in treatment of atherosclerosis. It should also have the potential to provide a therapeutic agent for treating various ocular and retinal disorders, degenerative muscle disorders, and for use in promoting neuronal or nerve regeneration. It may also be useful for protecting tissues against serious damage during radiation treatment.

In that bFGF and like growth factors obviously target the cell membrane signal-transducing receptors through which the biological activity is mediated, in developing the present invention it has been realised that in addition to the purpose of modulating biological activity and bringing about a biological effect, exogeneous growth factors can also be used as a carrier for other bioactive molecules that may act either as inhibitors or as stimulatory factors or as cytotoxic agents. This could for instance be particularly relevant to the treatment of certain tumours which are characterised by over-expression or up-regulation of FGF cell surface receptors.

In practice, however, the clinical use and administration of heparin-binding growth factors such as bFGF as therapeutic agents has hitherto been found to be subject to serious problems because the growth factors tend to be sequestered by becoming bound in the extracellular matrix (ECM) to heparan

sulphate proteoglycans (HSPG's) before reaching the target cells. They may also be susceptible to thermal inactivation, and to proteolytic degradation after attack for example by proteolytic enzymes.

This problem has been recognized particularly in relation to bFGF in the above-mentioned paper by Coltrini *et al* wherein it was pointed out that although theoretically the problem might be prevented by administering the growth factor together with free heparin, this is not a practicable solution because free heparin also reduces binding of bFGF to its cell surface signal-transducing receptors and thereby inhibits its biological activity. It was, however, also hypothesized in that paper that for efficient *in vivo* biodelivery, bFGF should be administered as a complex with an appropriately designed oligosaccharide that would protect the bFGF and prevent interaction with HSPG's of the ECM and safeguard against proteolytic degradation without affecting the interaction of the growth factor with its cell surface receptors so that its biological activity would not be inhibited. The way in which this objective could be achieved and the nature of the suggested complex was not described, however, and the suggestions made remained merely a generalised and indefinite hypothesis.

This problem of using exogeneous bFGF as a therapeutic agent is clearly applicable also to other heparin-binding proteins or polypeptides, native or synthetic, which are embraced by the term "growth factor" and which it may be desired to use and administer as therapeutic agents in the treatment of various disorders or diseases. A solution and practical guidance for overcoming the problem is, however, provided by the present invention.

ABBREVIATIONS

Throughout the present specification the following abbreviations are used:

GAG - glycosaminoglycan;

HS - heparan sulphate;

HSPG - heparan sulphate proteoglycan;

bFGF - basic fibroblast growth factor;

aFGF - acidic fibroblast growth factor;

dp - degree of polymerisation (e.g. for a disaccharide, dp=2, etc.);

GlcA - D-glucuronic acid;

IdoA - L-iduronic acid;

IdoA(2S) - L-iduronic acid 2-sulphate;

GlcNAc - N-acetyl D-glucosamine;

$GlcNSO_3$ - N-sulphated D-glucosamine;

$GlcNSO_3(6S)$ N-sulphated D-glucosamine 6-sulphate;

GlcA(2S) - D-glucuronic acid 2-sulphate;

ΔHexA - unsaturated uronic acid residue;

ΔGlcA - unsaturated hexuronate residue designed GlcA on the basis that it is believed to be derived from the saturated residue GlcA in an original polymer chain, e.g. based on the known specificity of heparitinase scission (see later);

The symbol (Δ) is used to indicate that the monosaccharide residue concerned may or may not be unsaturated, and the symbols (±2S) and (±6S) denote that a residue may or may not be sulphated at the C2 and C6 positions respectively.

## SUMMARY OF THE INVENTION

The present invention is based on a concept of forming a conjugate compound of a heparin-binding growth factor and a heparin or heparan sulphate oligosaccharide in which these components are linked together by covalent bonding, a result which it is believed has not hitherto been specifically reported.

By the term heparin or heparan sulphate oligosaccharide, as used in this specification, is meant an oligosaccharide, derived either from a natural source or made synthetically, having a saccharide sequence corresponding to a

sequence present in native heparin or heparan sulphate glycosaminoglycan, or derivable therefrom by chemical or enzymatic treatment.

Thus, in one aspect the present invention broadly provides bioactive material characterised in that it comprises a conjugate of a heparin-binding protein or polypeptide growth factor and a heparin or heparan sulphate oligosaccharide coupled together through covalent bonds.

In general, at least in preferred embodiments, said material is further characterised in that

(a) it is devoid of any significant binding affinity for heparin or for heparan sulphate glycosaminoglycans, or at least any such binding affinity is substantially less than that shown by said heparin-binding protein or polypeptide growth factor in a native unbound state, and

(b) in biological systems containing mammalian target cells having cell surface signal-transducing membrane receptors for said growth factor it retains a capacity to interact with said receptors and to modulate or exercise the normal biological activity of said growth factor.

With these conjugates, it has been found that notwithstanding the covalent bonding between the growth factor and the oligosaccharide the growth factor can still bind to its cell surface signal-transducing receptors on target cells, and even if this binding affinity is somewhat diminished it mimics at least to some extent that of the unbound or native growth factor. Also, these conjugates have been found to be resistant to proteolytic degradation and thermal inactivation. Depending on the oligosaccharide to which the growth factor is coupled, however, the effect of the covalent bonding may be either to stimulate or enhance the normal biological activity of the growth factor, or alternatively to inhibit such biological activity.

In practice, the binding affinity for heparin or heparan sulphate glycosaminoglycans of the covalently-bonded conjugates of the bioactive material provided by the present invention can be assessed for example by a filter binding assay as hereinafter described.

In preferred embodiments, the molecules of the oligosaccharide component of the conjugate are covalently coupled to molecules of the growth factor component through amide linkages, preferably to $C_6$ of iduronic acid (or glucuronic acid) residues of the oligosaccharide. Also, the molecules of the oligosaccharide component are in the form of linear chains of disaccharide units carrying one or more molecules of said growth factor component coupled along the length thereof.

The conjugates may be prepared by reacting the growth factor with an activated derivative of the oligosaccharide, e.g. a succinamide activated ester derivative, capable of reacting with a terminal amino group of a side chain of the growth factor. This side chain may be a side chain of a constituent amino acid, e.g. lysine, of the growth factor protein or polypeptide molecule, or it may be an intermediate spacer linkage between the growth factor and oligosaccharide.

Whether or not such intermediate spacer is provided can be optional. Without a spacer, cross-linking should only occur between oligosaccharides and amino acid side chains within the HS binding site of the growth factor.

The growth factor component may be provided by any of the following:

a fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (scatter factor), heparin-binding epidermal growth factor (HB-EGF), Interleukin (IL) 1α, 1β, 2, 3, 4, 6, 7, 8, 10 or 12, PDGF (platelet derived growth factor), TNFα (tumor necrosis factor), IGF I or II (insulin-like growth factor), IGFBP 3 or 5

(insulin-like growth factor binding protein), TGFβ (transforming growth factor), Interferon gamma (IFN-γ), Purpurin (retinal survival factor), Amphiregulin, Schwann cell mitogen, Pleiotrophin (p18), MSF (migration stimulating factor), HBNF (heparin-binding neurite promoting factor), NEL-GF (neu/erb B2 ligand-growth factor), MK (midkine factor), Platelet Factor-4 (PF4), GM-CSF, MIP (macrophage inflammatory protein) 1 alpha or 1 beta, SDMF (smooth muscle cell derived migration factor), GDNF (glial cell line derived neural growth factors), MDC (macrophage derived chemokine), BMP-2, NAP-2 (neutrophil activating peptide), endostatin, angiostatin and lymphotactin.

In preferred embodiments, however, the growth factor will usually be selected from the FGF family (e.g. aFGF or bFGF), VEGF, hepatocyte growth factor and HB-EGF.

The method of preparing the covalently cross-linked conjugates forms another aspect of the invention. In some preferred embodiments, the cross-linking process is a two step procedure, initially involving a brief incubation of HS oligosaccharide with a coupling agent such as 1-Ethyl-3-(3-dimethyl-aminopropyl)carbodiimide (EDC) in the presence of N-hydroxy succinamide (NHS). Together, the EDC and NHS may be termed "cross-linking reagents". This results in the conversion of some of the oligosaccharide carboxyls into succinamide esters. The reaction is terminated by neutralizing the EDC, e.g. by addition of β-mercaptoethanol, prior to addition of growth factor. Oligosaccharide-protein cross-linking arises from substitution of terminal amino groups, e.g. lysine ε-amino groups, of the protein for the succinamide ester. This two step process is illustrated diagrammatically in FIGURE 1 of the accompanying drawings and has the advantage that only the oligosaccharide comes into contact with the cross-linking reagents, i.e. the EDC and NHS, hence only oligosaccharide-protein cross-links are formed. This eliminates

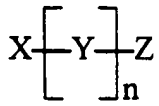
possible complications arising from protein-protein cross-linking which may occur if cross-linking reagents are exposed to a multicomponent system. The protocol therefore fulfils several criteria which favour active complex formation, in particular (i) direct oligosaccharide cross-linking into the growth factor binding site, and (ii) lack of inter- and intra-molecular protein-protein cross-links which could perturb further interaction of the complexes with FGFRs.

On the other hand, it has been found that the yields obtained in this two-step procedure are generally rather low and much higher yields can be obtained with a one step procedure using the coupling reagents EDC and S-NHS and a prolonged incubation period. In fact, given optimum conditions there are possibilities of achieving substantially 100% conversion of the growth factor to cross-linked HS oligosaccharide conjugates. In practice, this one-step procedure will therefore often be the preferred option.

The product may finally be purified so that it is substantially free of unbound or native growth factor, but since unreacted components associate strongly and co-elute with cross-linked complexes during gel filtration chromatography it may be necessary to raise the ionic strength substantially, e.g. by adding 2M NaCl to the buffer system.

For producing conjugates that can be incorporated in pharmaceutical compositions for therapeutic use in modulating the biological activity of the heparin-binding growth factor, the oligosaccharide may be selected so as to have a stimulatory effect on the biological activity of the growth factor component, or it may have an inhibitory effect on activity of the the growth factor component. Which of these two effects is obtained will generally depend on the size and composition of the oligosaccharide, including its sulphation pattern. In the case of bFGF for example, for binding to and stimulating activity of the growth factor there is evidence to suggest that the

oligosaccharides will generally be composed predominantly of a molecular species:



in which

X is ΔHexA(±2S)-GlcNSO$_3$(±6S),

Y is IdoA(±2S)-GlcNSO$_3$(±6S),

Z is IdoA-GlcR(±6S) or

IdoA(2S)-GlcR(±6S)

where R is NSO$_3$ or NAc, and

n is in the range of 3 to 7

If, however, the oligosaccharide is shorter, i.e. n is less than 3, or has a sulphation pattern that does not conform to the above, it may still bind to the growth factor in many cases this will occur without resulting in any stimulatory biological activity and the oligosaccharide may even act as an inhibitor of the biological activity of the growth factor. This is further discussed, for example, in the previously mentioned paper of Jayson and Gallagher.

In practice, the oligosaccharides used will usually be composed of less than 20 monosaccharide residues, although in some cases the oligosaccharides may be somewhat larger, e.g. up to 30 monosaccharide residues.

Although most of the experimental work has been carried out with bFGF, at least in principle the results should apply equally to other heparin-binding growth factors that are dependent on HS, e.g. as listed earlier. Thus, the direct covalent linking of the oligosaccharide to the growth factor can be expected generally to provide better bioavailability and enhanced targeting to the cell surface receptors when administered for therapeutic purposes, primarily as a result of reduced non-specific binding to extracellular matrix heparan

sulphates and reduced proteolytic degradation giving a longer growth factor half life.

From another aspect the invention can be defined as providing a method of modifying a heparin-binding growth factor to improve its suitability for administration to a mammal in the course of therapy, said method comprising coupling said growth factor to a heparin or heparan sulphate oligosaccharide to form a covalently cross-linked conjugate.

As already indicated, the invention also provides pharmaceutical formulations for therapeutic use in modulating the activity of a heparin-binding growth factor in the course of therapeutic treatment of a mammal, such formulations comprising a composition of bioactive material as hereinbefore defined in admixture with a pharmaceutically acceptable carrier therefor and/or a pharmaceutically acceptable additive, diluent or excipient.

Bioactive material in accordance with the invention wherein the oligosaccharide component promotes or stimulates biological activity of the growth factor component, especially as an active FGF-activity stimulating agent for promoting healing or tissue repair, may be used in treating mammals in need of such treatment for a variety of conditions requiring tissue repair, such as for example wound healing, bone healing, nerve regeneration, duodenal or venous ulcers, various ocular and retinal disorders, ischaemia, or may be used for protecting tissues against serious damage during radiation treatment. Where in contrast the bioactive material acts to inhibit biological activity of the growth factor component, especially for example as an effective FGF-activity inhibiting agent for controlling or reducing cell growth or proliferation, it may be used in treating mammals in connection with conditions or disorders such as diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g.

Herpes Simplex type 1), restenosis following angioplasty and other conditions where there is a requirement to inhibit FGF growth factor activity.

The further aspect of the present invention which is of major importance is that of using the covalently linked conjugates as carriers of cytotoxic drugs or other therapeutic molecules which can be linked to the oligosaccharide component, especially for targeting to cells where there is a high level of expression of the growth factor receptors for example.

Linking of steroid drug molecules, in particular cortisol, to the polysaccharide chains of heparin to produce conjugates that can act as angiogenesis inhibitors and exhibit antitumour activity has been disclosed in a paper entitled "Heparin-Steroid Conjugates: New Angiogenesis Inhibitors with Antitumour Activity in Mice" by P. E. Thorpe *et al* (1993), *Cancer Research*, 53, pp 3000-3007. According to this further aspect of the present invention a drug such as a cytotoxic agent or other therapeutically active agent is linked to the oligosaccharide component of a covalently linked oligosaccharide/growth factor conjugate so as to be targeted specifically to cells expressing receptors for the growth factor. In the preferred synthetic strategy, unlike the arrangement disclosed in the above-mentioned paper of Thorpe *et al* wherein the steroid was attached to numerous sites on the heparin molecule, the drug is attached at the reducing end of the oligosaccharide, preferably through a spacer arm or linkage, so that it should not interfere with any oligosaccharide/protein interactions or binding to the growth factor receptors.

Again, the oligosaccharide component may be selected so as to promote biological activity of the growth factor, e.g. in the case of bFGF an oligosaccharide wherein dp $\geq$ 12, or in some cases dp=10, or it may be selected so as to act as an inhibitor of growth factor activity, e.g. it may be an oligosaccharide which is less than a decasaccharide in length or which comprises a longer sequence lacking key O-sulphate groups.

As will be appreciated, the drug may of course be attached in the form of a prodrug derivative, and in a specific example that has featured in preliminary development work a water-soluble prodrug form of the tubulin assembly inhibitor drug Combretastatin A4 has been attached via a spacer arm to covalently coupled conjugates of bFGF and size-defined HS oligosaccharides. The synthetic strategy as used for synthesising the Combretastatin A4 prodrug derivative may involve the formation of a hydrazido-adipyl-azo HS oligosaccharide in order to form part of the spacer arm and to introduce a primary amino group to the reducing end of the oligosaccharide. Alternatively, a glycosylamine derivative of the HS oligosaccharide may be produced. A heterobifunctional agent N-succinimidyl (4-iodoacetyl)aminobenzoate may then be used to link the prodrug to the oligosaccharide hydrazide or glycosylamine derivative. This requires the introduction of a sulphydryl group on the prodrug, but this can be achieved by removal of a BOC protecting group and reacting the exposed amino group with N-succinimidyl S-acetylthioacetate. Overall, the conjugation is advantageously performed in two steps by first forming the iodoacetyl activated oligosaccharide hydrazide or glycosylamine derivative, followed by the subsequent addition of the sulphydryl deprotected prodrug. The resulting conjugates can then be purified by size exclusion HPLC for example.

It will be understood that where reference is made in this specification to a drug, this term is intended to extend also to other pharmaceutically acceptable bioprecursors (prodrug forms) where the context so admits. The term "prodrug" is used in the present specification to denote modified forms or derivatives of a pharmacologically active compound which biodegrade *in vivo* and become converted into said active compound after administration, especially oral or intravenous administration, in the course of therapeutic treatment of a mammal. Such prodrugs are commonly chosen because of an enhanced solubility in aqueous media which helps to overcome formulation

problems, and also in some cases to give a relatively slow or controlled release of the active agent. The term "drug" is furthermore intended also to embrace any therapeutically active agent that it may be desired to administer to a mammal in carrying out medical or therapeutic treatment.

From yet another aspect the invention can also be defined as providing a method of modifying a drug or prodrug to facilitate administration to a mammal and targetted delivery to cells having specific growth factor receptors, said method comprising coupling molecules of said drug or prodrug to molecules of a heparin or heparan sulphate oligosaccharide which in turn are covalently coupled or cross-linked to molecules of the specific growth factor that interacts with said receptors.

The invention also provides a method of manufacturing a medical preparation for administering to a mammal for carrying out targetted drug delivery in the course of therapeutic treatment, said method comprising mixing with a compatible pharmaceutically acceptable additive, carrier, diluent or excipient a bioactive material as hereinbefore defined which contains a therapeutically effective amount of a drug (or prodrug) linked to the oligosaccharide component.

The invention also provides a pharmaceutical composition or formulation for use in controlling the activity of fibroblast growth factors in mammals either for promoting tissue repair or for inhibiting cell growth or proliferation in the treatment of disorders resulting therefrom, said composition or formulation comprising a therapeutically effective amount of bioactive material as hereinbefore defined.

In forming the covalently cross-linked oligosaccharides/growth factor conjugates of the present invention, instead of reacting the growth factors with a succinimide ester activated oligosaccharide other means of achieving the cross-linking can be used. In particular, it has been found that covalent cross-

linkages can conveniently be formed by mixing a growth factor such as bFGF for example with a photoreactive oligosaccharide and illuminating with ultra-violet light.

By way of further explanation so that the skilled person in the art will more readily be able to appreciate the nature of the invention and will more readily be able to put it into practical effect, there now follows a fuller description of the invention, including some specific examples, background experimental work carried out by the inventors and various practical details thereof. In connection with this description, reference should also be had to the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

In said drawings,

FIGURE 1 is diagram illustrating the two-step process previously referred to for preparing the covalently cross-linked conjugates of this invention;

FIGURES 2A and 2B are diagrams illustrating the steps in a method of preparing an iodoacetyl-activated hydrazido-adipyl-azo oligosaccharide for use in coupling to a drug molecule;

FIGURES 3A and 3B are diagrams illustrating the steps in a method of preparing an iodoacetyl-activated N-glycyl-β-oligosaccharide for use in coupling to a drug molecule;

FIGURE 4 is a diagram illustrating the synthesis of a sulfydryl-containing Combretastatin A4 prodrug;

FIGURES 5A and 5B are diagrams showing Combretastatin A4/oligosaccharide conjugates produced with the oligosaccharide derivatives of FIGURES 2B and 3B respectively;

FIGURES 6 and 7 are diagrams showing various compounds that have been prepared during development work based on unsulphated HS derived disaccharides;

FIGURES 8A and 8B are diagrams illustrating the steps in a method of preparing a succinimide ester activated fluorescent tagged hexasaccharide ready for covalently coupling or cross-linking with bFGF;

FIGURES 9A and 9B are diagrams showing steps in a method of synthesising photoreactive oligosaccharides;

FIGURE 10 comprises two diagrams A and B that provide evidence from a filter binding assay of covalent cross-linking of bFGF-oligosaccharide conjugates and lack of binding affinity thereof for endothelial HS;

FIGURE 11 comprises two diagrams A and B that provide evidence from a filter binding assay of covalent cross-linking of photoreactive oligosaccharides with bFGF; and

FIGURES 12 to 16 are diagrams showing the results of filter binding assays for a number of different heparin-binding growth factors within the FGF family when treated with cross-linking reagents in the absence of, and in the presence of, dp12 HS oligosaccharides.

## DETAILED DESCRIPTION and EXAMPLES

For putting the present invention into practice suitable oligosaccharides must be prepared and by way of example there is described under EXAMPLE I below the preparation from porcine mucosal heparan sulphate of 12-mer oligosaccharides used in the development work on the invention. Alternative methods of preparation of covalently bound cross-linked conjugates of the 12-mer HS oligosaccharides with human recombinant bFGF

(supplied by R+D Systems, Abingdon, U.K.) are described under EXAMPLE II, including purification, testing and analysing the properties of the product.

The results of experiments to test the ability of a range of different heparin-binding growth factors to form covalently bound conjugates assessed by means of filter binding assays are described under EXAMPLE III. Although the growth factors tested in this example are all members of the FGF family of growth factors these results are considered to be a good indicator of the ability of heparin-binding growth factors generally to form covalent cross-linked conjugates with HS oligosaccharides of suitable size and sulphation pattern.

In connection with the proposed use of the covalently bound cross-linked heparin or heparan sulphate oligosaccharide and heparin-binding growth factor conjugates of the present invention as carriers for targeted delivery of therapeutic drugs, by way of example there are described under EXAMPLE IV various stages showing how an isoleucine derivative prodrug form of the cytotoxic anticancer drug Combretastatin A4 can be coupled to an oligosaccharide that is then covalently linked with a heparin-binding growth factor, e.g. bFGF, in accordance with the invention.

For this purpose, two main synthetic methods are proposed for linking the Combretastatin drug molecule to the oligosaccharide at the reducing end of the latter via a neutral spacer arm. The first method illustrated in Figures 2A and 2B involves the formation of hydrazido-adipyl-azo HS oligosaccharides as exemplified by structure **3**. This introduces a primary amine to the oligosaccharide and also helps to form part of the required spacer arm. The second method, illustrated in Figures 3A and 3B, involves the formation of a oligosaccharide glycosylamine derivative structure **6**. This has desirable properties in that it keeps a cyclic structure and mainly adopts a β- anomeric configuration. Unfortunately these glycosylamine derivatives are readily hydrolysed so to improve stability they are converted to a relatively stable N-glycyl-β-derivative (structure **8**). The two oligosaccharide derivatives are then

converted by addition of N-succinimidyl(4-iodoacetyl)aminobenzoate (SIAB) to iodoacetyl-activated oligosaccharides (structures **4** and **9**) which can readily react with free sulphydryl groups. Such sulphydryl groups can be introduced into the Combretastatin prodrug as shown in Figure 4. This involves the reaction of the isoleucine prodrug derivative of Combretastatin (structure **10**) with N-succinimidyl S-acetylthioacetate (SATA) to form a protected sulfydryl derivative of the drug (structure **11**). Deprotection of this derivative is then achieved by treatment with hydroxylamine to give structure **12** which can then be linked to the oligosaccharide by addition to the iodoacetyl-activated oligosaccharides. The resulting compounds (structures **13** and **14**) are shown in Figure 5. These can then be complexed subsequently to form covalent bonded growth factor/oligosaccharide/drug conjugates. It may be noted that the compounds of these structures **13** and **14** may be useful therapeutic agents in their own right. In both the two synthetic methods described it will be seen that the drug is attached at the reducing end of the oligosaccharide, and so will not interfere with any oligosaccharide-protein interactions. The above derivatization methods were tested during the development work using, for the sake of economy, unsulphated HS derived disaccharides. Thus, Figure 6 and Figure 7 show the compounds that were actually made in this development work, including the sulfydryl-containing combretastatin prodrug. Having established the derivatization protocol by use of disaccharides, however, the same methodology can readily be applied to longer oligosaccharides.

For establishing that the oligosaccharides are in fact covalently attached to the growth factor protein during cross-linking, and that molecules such as cytotoxic drugs can be attached to the oligosaccharides without affecting their biological activity, or their ability to cross-link to the growth factor, fluorescent tagging of the oligosaccharide is useful. The reaction scheme for the fluorescent labelling of HS hexasaccharides with 2-aminobenzoic acid (structure **25**) is described under EXAMPLE V and is

illustrated in Figures 8A and 8B. Since the oligosaccharide alone is not fluorescent, only tagged material can be detected by fluorescent visualisation. The tagged oligosaccharide is then converted into a succinimide ester structure **26** and is cross linked to bFGF. During the preliminary development work using this technique, when the reaction mixtures were run on SDS PAGE gels, they clearly showed the presence of a fluorescent band in the position where the bFGF/oligosaccharide conjugates are normally located, and the intensity of this band increased with the amount of hexasaccharide used in the cross-linking reaction. In contrast, tagged oligosaccharide alone and bFGF alone showed no such fluorescent bands in this position, demonstrating that it is possible to place other molecules such as cytotoxic drugs on the reducing end of the oligosaccharides without affecting their ability to cross-link to the growth factor.

EXAMPLE VI describes experiments that were performed to verify that cross-linking between oligosaccharides and bFGF could be achieved using cross-linkers other than succinimide esters and shows how a photoreactive compound 4-(p-Azidosalicylamido)butylamine (ASBA) attached to the carboxylic acid group of internal Iduronic acid containing disaccharides can produce a photoreactive oligosaccharide which, when mixed with bFGF and illuminated by U.V. light, will form covalent cross-linkages.

## EXAMPLE I

### Preparation of DP12 Oligosaccharides

100mg of porcine mucosal HS (obtained from Organon, Oss, The Netherlands) in 0.5ml of heparinase buffer (100mM sodium acetate, 0.1mM calcium acetate pH 7.0), was incubated initially with 0.25iu of the enzyme heparinase III (EC 4.2.2.8- obtained from Grampian Enzymes, Orkney, U.K.)

followed by two further additions after 24 and 48 hours. The digest was monitored by absorbance at 232nm until no further increase occurred. Oligosaccharides produced by this enzymatic digestion were resolved by gel filtration size exclusion chromatography using a Biogel-P6™ column (1.5 x 170cm) in 0.5M $NH_4CO_3$ at a flow rate of 6ml/h. 1ml fractions were collected and oligosaccharides detected by monitoring the absorbance at 232nm. Size defined oligosaccharides in the range dp2-dp16 were pooled, freeze dried and stored at -80°C until required.

## EXAMPLE II

### Preparation of Covalent Cross-Linked Conjugates with bFGF

#### A. Method Using One Step Cross-Linking Procedure

A one step cross-linking procedure was carried out by incubating human recombinant bFGF growth factor (10μM), obtained from R+D Systems, Abingdon, Oxon, U.K., and dp12 HS oligosaccharides (40μM) simultaneously with 20mM EDC and 12mM S-NHS in 0.1M NaCl, 0.1M MES, pH 6.0 for 2 hours at 25°C. The reaction was then terminated by the addition of β-mercaptoethanol to a final concentration of 20mM (to neutralise EDC) and hydroxylamine to a final concentration of 50mM (to hydrolyse unreacted succinimide esters). These conditions were chosen since they resulted in the complete conversion of free growth factor to conjugates without protein-protein oligomerization.

#### B. Alternative Method Using Two Step Cross-Linking Procedure

This was carried out according to the scheme of Figure 1. Briefly, dp12 oligosaccharides (3μg) from Example I in 40μg coupling buffer, namely 0.1M NaCl, 0.1MES at pH 6.0, were incubated for 15 minutes at 25°C with EDC (6mMoles) and S-NHS (15mMoles) (from Pierce, Chester, U.K.). The reaction was terminated by addition of β-mercaptoethanol to a final

concentration of 20mM. The oligosaccharides, so-activated, were then added to a 50μM solution of human recombinant bFGF in coupling buffer to give a bFGF:dp12 molar ratio of 1:4. This reaction mixture was then incubated for 2 hours at 25°C.

Products from each of the above methods were analysed to determine the extent of conjugate formation by standard 12% SDS polyacrylamide electrophoresis (SDS PAGE) and western blotting, followed by enhanced chemiluminescence (ECL) immunodetection using monoclonal anti human basic fibroblast growth factor rabbit immunoglobulin (see below) prior to purification.

In order to optimize yields, the ratio of bFGF:oligosaccharide can be varied, and also it should be noted that the ratio of EDC to S-NHS can affect coupling efficiency. The ratios quoted in the above example, i.e. a 1:4 ratio of bFGF:dp12 and EDC/S-NHS concentrations of 20mM and 12mM respectively for the one step procedure, and 6mM and 15mM respectively for the two step procedure, represent the ratios chosen in a typical case to achieve optimal cross-linking conditions.

Immunodetection of bFGF

Western blots of SDS PAGE gels on nitrocellulose membranes were blocked overnight with 1% (w/v) BSA in PBS and then washed three times with PBS, 0.05% (v/v) Tween 20. The membrane was then incubated with a 1:200 dilution of rabbit anti human bFGF monoclonal antibody for 2 hours at 4°C. The antibody solution was then removed and the membrane washed three times with PBS, 0.05% (v/v) Tween 20. The membrane was then further incubated with a 1:1000 dilution of horseradish peroxidase conjugated swine anti-rabbit IgG for 1 hour. The membrane was then finally washed eight times in succession with PBS, 0.05% (v/v) Tween 20 and the presence of bFGF

visualised by ECL (Amersham Life Science Ltd., Bucks. U.K.) following the manufacturer's protocol.

Purification of bFGF-Oligosaccharide conjugates

1st Method

Products of the cross-linking reaction were purified to homogeneity by gel-filtration chromatography with two Superose 12™ columns (Pharmacia Biotech. St. Albans, Herts., U.K.) in series. More particularly, cross-linked samples were applied to the two Superose 12™ columns linked to a Gilson HPLC system, equilibrated in 50mM phosphate buffer containing 2M NaCl, pH 7.4. The samples were eluted at a flow rate of 0.5ml/min. Fractions (250µl) were collected and the elution profile was monitored by absorbance at 280nm. Fractions containing protein were desalted by microdialysis (MW cut-off 12,000 daltons) against 5mM Tris-HCl, pH 7.4, at a flow rate of 1.5ml/min overnight at 4°C. Desalted samples were analyzed for purity by 12% SDS-PAGE followed by western blotting and ECL immunodetection; fractions containing cross-linked bFGF monomers and bFGF dimers were pooled separately and, in some cases, were then reapplied to the columns as above. Finally, the purified components were lyophilized and stored at -80°C until required. The 2M NaCl was included in the buffers to minimize interactions with free growth factor and oligosaccharide and so reduce contamination of the conjugates, but as some contamination may still occur the subsequent pooling of the samples and reapplication to the column was intended to ensure that all traces of free growth factor would finally be removed.

It should be appreciated that at least the initial products of cross-linking may often comprise not only monomers of the growth factor bound to the oligosaccharide molecules but also dimers or larger oligomers of the growth factor. At least in the case of bFGF, such dimers have been identified as being comprised of two disulphide-bonded bFGF monomers and the amount of

dimers formed, if any, may depend on the reaction conditions. The latter, and especially the ratio of the coupling reagents, will also generally affect coupling efficiency. If should be noted that although the two-step cross-linking procedure can be advantageous in reducing likelihood of protein-protein cross-linking occurring, the cross-linking efficiency is generally low and the majority of the growth factor remains in the native form, unlike in the one-step cross-linking procedure which can result in substantially all the growth factor being cross-linked.

2nd Method

The above disclosed purification method was not entirely satisfactory since apart from the fact that it was time consuming (several days), it could also result in the loss of approx. 90% of products due to adsorption to surfaces. An alternative and preferred purification method utilises hydrophobic interaction chromatography (HIC) which is a very gentle method of separating proteins by their hydrophobic nature (typical reverse phase chromatography results in the complete loss of bFGF biological activity, due to protein denaturation). This method is a valuable tool for conjugate production since it is very fast and results in high yields of active conjugates. It is also useful for removal of excess oligosaccharides since only the growth factor is retained by the column under the initial conditions used because the highly charged oligosaccharides wash straight through. The method however, relies on the complete conversion of growth factor to monomer conjugate so that it will generally be necessary to use the previously described one step cross-linking procedure or protocol. On the other hand, using this method for the purification of at least bFGF/oligosaccharide monomer conjugates, the yield or recovery of product has been found to reach between 80-90% or more of the applied sample.

In this particular example using bFGF and dp12 HS oligosaccharides in the one step cross-linking procedure, after analysing the reaction products by SDS 12% PAGE to determine the extent of conjugate formation, the products

of reactions that had gone to completion were then purified by HIC. Briefly, the conjugate preparations produced were applied to a Phenyl Superose™ HR5/5 column in 2.0M $(NH_4)_2SO_4$, 50mM phosphate buffer pH 7.4 at a flow rate of 0.5ml/min. The column was then washed for 15mins with $(NH_4)_2SO_4$, 50mM phosphate buffer pH 7.4 and bound FGF complexes were eluted using a 10min linear gradient of 2.0-0.0M $(NH_4)_2SO_4$, in 50mM phosphate buffer pH 7.4, again at a flow rate of 0.5ml/min. Fractions were collected every 2mins, and were desalted using a Hightrap™ desalting column prior to SDS 12% PAGE analysis and immunodetection.

Biological Activity Testing of bFGF-Oligosaccharide Conjugates

A BaF-cell assay has been used for testing biological activity.

BaF3 cells are a lymphoblastoid line which do not express FGFR's and fail to respond to bFGF. These cells were transfected with FGFR to produce a clone of cells designated F32 cells. These F32 cells are devoid of functional cell surface HS, and only respond to bFGF in the presence of added HS or heparin. Addition of bFGF monomer conjugates to the assay system showed the complexes represented by these conjugates to be active with dose dependant activity (measured by $[^3H]$ thymidine incorporation) reaching a plateau at levels equivalent to maximum stimulation with intact HS and bFGF, although the dose of conjugate needed to bring about maximum stimulation was somewhat higher than that of the native growth factor.

The F32 cells used were routinely maintained in RPMI-1640 medium, 10% horse serum supplemented with IL-3 conditioned medium at 37°C, 5% $CO_2$. Conditioned medium was prepared from WEHI 3b cells.

For the assay system F32 cells were plated into 96-well plates at a density of 50,000 cells/well in 100µl RPMI-1640 medium, 10% horse serum supplemented with cross-linked conjugates over a range of concentrations. Cells were incubated for 46 hours before addition of $^3H$-thymidine

(0.3μCi/well) for a further 2 hours. Incorporation of thymidine was stopped by harvesting cells on a Filtermate-196™ cell harvester. Cells were allowed to air dry before addition of 25μl of Microscint O™ to each well. Radioactivity incorporated was then counted on a scintillation counting top count system.

Analysis of the affinity of Cross-linked Conjugates for HS/Heparin

The binding affinity of the bFGF-oligosaccharide conjugates produced with respect to extracellular heparin or heparan sulphate was checked by two methods, (1) a filter binding assay method, and (2) a heparin agarose bead assay, as described below.

(1) Filter binding Assay Method

The assay was performed using a membrane filtration apparatus (Millipore, Watford, Herts., U.K.). Briefly, 4μg of native growth factor or growth factor/HS oligosaccharide conjugate material were applied to nitrocellulose membrane filters in binding buffer (10mM Tris-HCl, pH 7.3). The filters were then washed with 10ml of 2M NaCl in binding buffer to remove any non cross-linked oligosaccharide that may have been present, and were then re-equilibrated by washing with binding buffer. Radiolabelled $^{3}H$-heparan sulphate was then applied in 5ml of binding buffer and cycled through the filters three times. Thereafter, the filters were washed with 10ml of binding buffer to remove unbound material, and bound HS was released by sequential washing, first with three 5ml aliquots of 0.3M NaCl followed by three 5ml aliquots of 2.0M NaCl in binding buffer. 5ml fractions were collected and radio-labelled eluted material was quantified by scintillation counting.

These filter binding experiments were performed on both native bFGF and monomer conjugate, and the results showed the complex to have no high or low affinity binding capacity for HS, as indicated by the diagrams A and B of

Figure 10, diagram A relating to native bFGF and diagram B relating to cross-linked bFGF/dp12 monomers. This also indicates that the oligosaccharide in the conjugates is covalently linked into the growth factors HS binding site, resulting in the site being completely obscured from further HS interactions.

(2) Heparin Agarose Bead Assay

Samples (100ng) of native bFGF and bFGF/dp12 monomer conjugates in 1ml of binding buffer 50mM phosphate buffer containing 1% BSA and 0.1% Tween 20 were added to 50µl of heparin agarose beads which had previously been washed 3 times with 1ml of binding buffer. The samples were incubated overnight at 4°C with end over end mixing. Supernatants were removed and the beads washed twice with binding buffer before the addition of 1ml of binding buffer containing appropriate concentrations of NaCl. This was followed by incubation for 1 hour at room temperature with end over end mixing. This step was repeated three more times to completely remove unbound growth factor from the beads. Bound material was removed from the beads by solubilizing the 30µl of SDS-PAGE sample treatment buffer. Proteins were resolved by SDS-PAGE followed by western blotting and visualisation by immunodetection and ECL.

The results demonstrated an inability of the conjugates to bind to heparin immobilised on agarose beads, again showing the conjugates to have no high or low affinity binding capacity for HS, indicating that the oligosaccharide was covalently linked onto the growth factors HS binding site resulting in the site being completely obscured from further HS interactions.

It was also found that no conjugates were formed when the cross-linking procedure was performed using denatured protein, indicating that the cross-linking reaction was specific for the HS binding site/sites on the growth factor and was not the result of random molecular collisions.

Stability of bFGF/HS Cross-linked Conjugates

It has been previously reported that heparin protects bFGF and aFGF from proteolytic degradation. To test whether this is the case for the conjugates, dp12/bFGF cross-linked monomer conjugates and native bFGF were treated with trypsin, and the degradation products examined by SDS PAGE analysis and immunodetection. More particularly, basic FGF or dp12/bFGF monomer conjugates (0.5μg) were mixed in 50μl PBS containing 1% of the detergent known as CHAPS (3-[(cholamidopropyl)-dimethylammonio]-1-propane-sulfonate) before addition of 4μl trypsin (2mg/ml). The reaction mixtures were then incubated for 24h at 37°C, aliquots were then removed and analysed by SDS 12% PAGE and immunodetection. It was found that the dp12/bFGF cross-linked monomer conjugates were significantly more resistant to proteolytic degradation when compared to the native bFGF, which was extensively degraded.

Thus it was demonstrated that covalently cross-linking dp12 HS oligosaccharides to bFGF results in the formation of complexes which are not only biologically active but which are also more stable to proteolytic degradation than the native growth factor. Again, it is believed that this finding applies generally to similar cross-linked complexes or conjugates involving other heparin-binding growth factors and/or other HS oligosaccharides although in the latter case the size range required for maintaining biological activity is likely to vary with the particular growth factor concerned.

## EXAMPLE III

## Cross-Linking of HS Oligosaccharides and other Heparin-binding Growth Factors

Various other heparin-binding growth factors have also been assessed for their ability to form covalently-bound conjugates with HS oligosaccharides, this being determined by both SDS-PAGE analysis and filter binding assays.

Figures 12 to 16 show filter binding assay results for several members of the FGF family, namely aFGF, bFGF, FGF-4, FGF-6 and FGF-9. When these proteins were treated with cross-linking reagents (EDC and S-NHS) in the absence of oligosaccharide no differences were seen in the ability of the growth factors to bind [$^3$H]HS. However, when dp12 HS oligosaccharides were included in the reaction mixture [$^3$H]HS binding was either significantly reduced (FGF-4, FGF-9) or completely blocked (aFGF, bFGF, FGF-6). Again, this is believed to be a result of HS oligosaccharide/growth factor conjugate formation with the oligosaccharide being cross-linked into the HS binding site. As with bFGF, the cross-linking reaction products obtained with aFGF, FGF-4, FGF-6, and FGF-9 proteins should similarly be biologically active.

**EXAMPLE IV**

**Preparation of Conjugated Combretastatin/Oligosaccharides**

Section IV.1 (Stage 1 of Method A) - Synthesis of Hydrazido-adipyl-azo HS oligosaccharides (Figures 2 and 6 - Structures 3 and 16)

HS disaccharides (structure **15**) (5mg) were dissolved in formamide (1ml) at 50°C, adipate dihydrazide (10mg) was added, and the reaction mixture maintained at 50°C for 24 hours. The reaction products were then purified by Bio-Gel P-2 chromatography (1cm x 120cm) in 0.5M ammonium bicarbonate at a flow rate of 6ml/h. Elution was monitored online by U.V. absorbance at 232nm and 1ml fractions were collected. Fractions containing hydrazido-adipyl-azo HS disaccharides (structure **16**) were pooled and freeze dried.

Section IV.2 (Stage 1 of Method B) - Synthesis of Glycosylamines (Figures 3 and 7 - Structures 6 and 19)

Tri-sulphated HS disaccharides (structure **18**) (10mg) were dissolved in 0.5ml of water and 1g of ammonium bicarbonate was added followed by heating at 50°C for 24 hours. The formation of glycosylamine (structure **19**) and glycosylamine-carbonate was monitored by SAX-HPLC (Spherisorb S5-

SAX column), eluted at 1ml/min with milli Q water, pH 4.0, and a gradient of NaCl 0-1M in 40 minutes. Excess ammonium bicarbonate was removed by repeated freeze drying, and after each cycle of freeze drying the conversion of glycosylamine-carbonate to glycosylamine was monitored by SAX-HPLC. Typically around 4 cycles of freeze drying were required for complete removal of bicarbonate and conversion of the disaccharide glycosylamine-carbonate to the glycosylamine form.

Section IV.3 (Stage 2 of Method B) - Chloroacetylation of Glycosylamines (structures **6** and **19**) to Form Stable N-Glycyl-β-Oligosaccharides (structures **8** and **21**)

Glycosylamines (5mg) were resuspended in 100µl of 1M sodium bicarbonate followed by the addition to a 10 fold molar excess of chloroacetic anhydride. The pH was monitored and additional base was added if the pH dropped below 7.0. After 2 hours at room temperature another aliquot of base and of the anhydride was added. The mixture was applied to a Bio-Gel P-2 column (as described previously) to remove excess chloroacetic anhydride, and oligosaccharide containing fractions were freeze dried. The mixture was then stirred in saturated ammonium carbonate at 50°C in a sealed tube for 24 hours, and the N-Glycyl-β-oligosaccharide (structure **21**) was then purified by Bio-Gel P-2 chromatography and freeze dried to remove the bicarbonate.

Section IV.4 (Next Stage for Methods A and B) - Iodoacetyl Activation of HS oligosaccharides (structures **4, 9 and 17, 22**)

Hydrazido-adipyl-azo HS oligosaccharides (structures **3** and **16**) (1mg) or N-Glycyl-β-oligosaccharides (structures **8** and **21**) (1mg) were dissolved in phosphate buffer pH 7.5 (100µl) to which N-succinimidyl(4-iodoacetyl)-aminobenzoate (SIAB) (0.25mg) was added in DMSO (200µl). The reaction was allowed to proceed for 2 hours at 50°C. The products (structures **4**, **9**, **17**,

22) were then desalted using a Presto™ desalting column, equilibrated in 50mM borate buffer, pH 8.3, with 5mM EDTA. Fractions containing iodoacetyl activated HS oligosaccharides were pooled prior to addition of the sulfydryl derivative of Combretastatin.

Section IV.5 - Synthesis Combretastatin Prodrug Sulfydryl Derivative (structure 12)

Combretastatin-isoleucine prodrug (structure **10**) (2mg) was added to N-succinimidyl S-acetylthioacetate (SATA) (2mg) in DMSO (1ml), and reacted for 2 hours at room temperature. The protected thiol derivative of Combretastatin (structure **11**) was purified by reverse phase chromatography (ODS 2 column) at a flow rate of 1m/min using a gradient of 50-100% methanol in water over 30 minutes. A deacylation solution was prepared consisting of hydroxylamine.HCl (1.74g) and EDTA (0.365g) in 50ml of 62.5mM phosphate buffer pH 7.5. The protected sulfydryl derivative of Combretastatin (0.5mg) was dissolved in 1ml of the deacylation solution, and the reaction was allowed to progress at room temperature for 2 hours. The de-protected sulfydryl containing Combretastatin derivative (structure **12**) was then used immediately.

Section IV.6 - Conjugation of Iodoacetyl Activated Oligosaccharides with Combretastatin Prodrug Sulfydryl Derivative (structures 13 and 14)

In this next stage, conjugation of iodoacetyl-activated oligosaccharides (structures **4** and **9**) is achieved by simply adding the deprotected sulfydryl Combretastatin derivative (structure **12**) to the pooled iodoacetyl-activated oligosaccharides formed in Section IV.4 and allowing the reaction to proceed at room temperature for 4 hours. Conjugated Combretastatin/oligosaccharides (structures **13** and **14**) are then purified by size exclusion HPLC.

Section IV.7 - Cross-linking of Combretastatin/oligosaccharide conjugates (structures **13** and **14**) to bFGF

This cross-linking stage is conveniently carried out by incubating the combretastatin/oligosaccharides conjugates (structures **13** and **14**) in coupling buffer 0.1M NaCl, 0.1MES, pH 6.0 for 15 minutes at 25°C with 6mM EDC and 15mM S-NHS. The reaction is terminated by addition of β-mercaptoethanol to a final concentration of 20mM. The activated oligosaccharides thus formed are then added to a solution of bFGF in coupling buffer to give a bFGF:oligosaccharide ratio of 1:4, and incubated for 2 hours at 25°C. The products can be analyzed by standard SDS polyacrylamide electrophoresis and western blotting followed by enhanced chemiluminescence (ECL) immunodetection using monoclonal anti human basic fibroblast growth factor rabbit immunoglobulin.

Section IV.8 Purification of Cross-linked Products

In a typical purification stage, cross-linked samples are applied to two Superose™ 12 columns linked to a Gilson HPLC system, equilibrated in 50mM phosphate buffer containing 2M NaCl, pH 7.4, and samples are eluted at a flow rate of 0.5ml/min. Fractions (150µl) are collected and the elution profile is monitored by absorbance at 280nm. Fractions containing protein may be desalted by microdialysis against 5mM Tris-HCl pH 7.4 at a flow rate of 1.5ml/min overnight at 4°C. Desalted samples can then be analyzed for purity by SDS-PAGE and ECL immunodetection, and fractions containing the cross-linked products are pooled and reapplied to the columns as above. Finally, the purified components are lyophilized and stored at -80°C until required.

## EXAMPLE V

### Fluorescent Labelling of Hexasaccharides and Cross-linking to bFGF

HS derived hexasaccharides (500µg) dried by evaporation were dissolved in 125µl of 400mM 2-aminobenzoic acid in formamide, followed by

the addition of 125µl of 200mM $NaBH_3CN$ also in formamide. The solution was mixed and allowed to react at 50°C for 17-24 hours. The fluorescently tagged hexasaccharides formed (structure **25**) were separated from free 2-amino benzoic acid using 2 High trap desalting columns connected together and equilibrated with dd $H_2O$ at a flow rate of 1ml/min. Samples were applied to the columns and the eluent was monitored using an online fluorescence detector (EX=310nm, EM=420nm) and 0.5ml fractions were collected. Fractions containing tagged hexasaccharide were pooled, freeze dried, and stored at -20°C in the dark until required. Basic fibroblast growth factor (0.5µg) was added to increasing amounts of tagged hexasaccharide to give tagged hexasaccharide:bFGF ratios of 1:4, 1:2, 1:1, 2:1, 4:1, 8:1, 16:1 and 32:1. Next, S-NHS (130ug) and EDC (1.7mg) was added and the reaction mixtures were made up to a 50µl volume in 0.1M MES, 0.1M NaCl buffer, pH 6.0. The cross-linking reaction was allowed to proceed for 2 hours, after which time samples were subjected to SDS-PAGE and the fluorescent band pattern was detected by U. V. illumination and photography.

## EXAMPLE VI

### Photoreactive Cross-linking of HS Oligosaccharides to bFGF

In this example, HS oligosaccharides (1mg), 7 disaccharides in length (dp14), S-NHS (1mg), EDC (0.5mg) and the photoreactive compound 4-(p-Azidosalicylamido)butylamine (ASBA) (1.3mg) were dissolved in 0.1M Mes, 0.1M NaCl buffer pH 6.0, and the reaction was allowed to proceed via the formation of a succinimide activated ester derivative (structure **27**) for 2 hours. The resulting photoreactive dp14 oligosaccharides (structure **28**) were then separated in the dark from excess reagents using a Hightrap desalting column equilibrated in dd $H_2O$. Fractions containing the activated oligosaccharides were pooled and freeze dried, and following this purification the photoreactive dp14 oligosaccharides (10µg) were added to bFGF (15µg) in 100µl of PBS, and left at room temperature for 15 minutes, followed by illumination for 10

minutes under U.V. light. Aliquots of the growth factor (5μg) were removed and the level of cross-linking evaluated by filter binding analysis using radiolabelled HS. The photoreactive aryl azide is unreactive in the dark but upon illumination forms an extremely reactive nitrene which can react with any residue on the protein. The degree of cross-linking was determined by a filter binding assay, and the results as illustrated in Figure 11 clearly show a dramatic drop in binding of radiolabelled HS to bFGF after exposure to photoreactive oligosaccharides and U.V. light, indicating that the photoreactive oligosaccharides had been cross-linked into the growth factor's binding site as seen with succinimide esters.

Therapeutic Use

As already indicated the bioactive material provided by the covalently cross-linked conjugates of the present invention can be useful as a therapeutic agent and for this purpose it can be used to make a medicament in the form of a pharmaceutical composition or formulation for administration to a mammal in need of treatment. Formulations of the present invention, for medical use, will generally comprise a preparation of said bioactive material together with one or more pharmaceutically acceptable carriers and, optionally, other therapeutic ingredients. The carrier(s) must be pharmaceutically acceptable in the sense of being compatible with other ingredients of the formulation and not deleterious to the recipient thereof.

Pharmaceutical formulations of the bioactive material include those suitable for oral, rectal, topical and parenteral (including subcutaneous, intramuscular, intravenous and spinal) administration, or for use in inhaler devices.

The formulations may conveniently be presented in unit dosage form, especially when the conjugates are used as drug carriers, and may be prepared

by any of the methods well known in the art of pharmacy. All methods include generally the step of bringing the bioactive therapeutic material into association with a carrier which constitutes one or more accessory ingredients. Usually, the formulations will be prepared by uniformly and intimately bringing the bioactive material into association with a liquid carrier or with a finely divided solid carrier or with both and then, if necessary, shaping the product into desired formulations.

Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets or lozenges, each containing a predetermined amount of the bioactive material; as a powder or granules; or a suspension in an aqueous liquid or non-aqueous liquid such as a syrup, an elixir, an emulsion or a draught. The bioactive material may also be presented as a bolus, electuary or paste.

A tablet may be made by compression or moulding of the bioactive material, generally with one or more accessory ingredients.

A syrup may be made by adding the bioactive material to a concentrated, aqueous solution of a sugar, for example sucrose, to which may be added any accessory ingredient. Such accessory ingredient(s) may include flavourings, an agent to retard crystallisation of the sugar or an agent to increase the solubility of any other ingredient, such as a polyhydric alcohol for example glycerol or sorbitol.

Formulations for rectal administration may be presented as a suppository with a usual carrier such as cocoa butter.

Formulations suitable for parental administration conveniently comprise a sterile aqueous preparation of the bioactive growth factor/HS oligosaccharide conjugate which is preferably isotonic with the blood of the recipient unless perhaps it is to be administered by spinal injection.

In addition to the aforementioned ingredients, pharmaceutical formulations of this invention, for example ointments, creams and the like, may also include one or more accessory ingredient(s) selected from diluents, buffers, flavouring agents, binders, surface active agents, thickeners, lubricants, preservatives (including antioxidants) and the like.

As will be seen, the invention presents a number of different aspects and it embraces within its scope all novel and inventive features and aspects herein disclosed, either explicitly or implicitly and either singly or in combination with one another. Also, many modifications are possible and, in particular, the scope of the invention is not to be construed as being limited by the illustrative examples or by the terms and expressions used herein merely in a descriptive explanatory sense.

CLAIMS

1. Bioactive material characterised in that it comprises a conjugate of a heparin-binding protein or polypeptide growth factor and a heparin or heparan sulphate oligosaccharide coupled together through covalent bonds.

2. Bioactive material as claimed in Claim 1 further characterised in that

(a) it is devoid of any significant binding affinity for heparin or for heparan sulphate glycosaminoglycans, or at least any such binding affinity is substantially less than that shown by said heparin-binding protein or polypeptide growth factor in a native unbound state, and

(b) in biological systems containing mammalian target cells having cell surface signal-transducing membrane receptors for said growth factor it retains a capacity to interact with said receptors and to modulate or exercise the biological activity of said growth factor.

3. Bioactive material as claimed in Claim 1 or 2 wherein molecules of said growth factor component are covalently coupled through amide linkages to iduronic acid or glucuronic acid residues within the molecules of the oligosaccharide component.

4. Bioactive material as claimed in any of the preceding claims wherein said amide linkages couple to $C_6$ of said iduronic acid or glucuronic acid residues.

5. Bioactive material as claimed in any of the preceding claims wherein the molecules of the oligosaccharide component are in the form of linear chains of disaccharide units carrying one or more molecules of said growth factor component coupled along the length thereof.

6. Bioactive material as claimed in any of the preceding claims wherein the covalent couplings between the molecules of the growth factor component

and the molecules of the oligosaccharide component involve side chains of constituent amino acids of the growth factor protein or polypeptide molecules.

7. Bioactive material as claimed in any of the preceding claims wherein the covalent couplings between the molecules of the growth factor component and the molecules of the oligosaccharide component include an intermediate spacer linkage between the growth factor and oligosaccharide molecules.

8. Bioactive material as claimed in any of the preceding claims wherein the growth factor component is one of the following:

a fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), hepatocyte growth factor (scatter factor), heparin-binding epidermal growth factor (HB-EGF), Interleukin (IL) 1α, 1β, 2, 3, 4, 6, 7, 8, 10 or 12, PDGF (platelet derived growth factor), TNFα (tumor necrosis factor), IGF I or II (insulin-like growth factor), IGFBP 3 or 5 (insulin-like growth factor binding protein), TGFβ (transforming growth factor), Interferon gamma (IFN-γ), Purpurin (retinal survival factor), Amphiregulin, Schwann cell mitogen, Pleiotrophin (p18), MSF (migration stimulating factor), HBNF (heparin-binding neurite promoting factor), NEL-GF (neu/erb B2 ligand-growth factor), MK (midkine factor), Platelet Factor-4 (PF4), GM-CSF, MIP (macrophage inflammatory protein) 1 alpha or 1 beta, SDMF (smooth muscle cell derived migration factor), GDNF (glial cell line derived neural growth factors), MDC (macrophage derived chemokine), BMP-2, NAP-2 (neutrophil activating peptide), endostatin, angiostatin and lymphotactin.

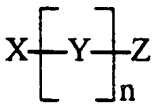
9. Bioactive material as claimed in Claim 8 wherein the growth factor is selected from the group consisting of aFGF and bFGF, VEGF, hepatocyte growth factor and HB-EGF.

10. Bioactive material as claimed in any of the preceding claims wherein

the molecules of the oligosaccharide component are composed of up to 30 monosaccharide residues.

11. Bioactive material as claimed in any of the preceding claims wherein the molecules of the oligosaccharide component are composed of less than 20 monosaccharide residues.

12. Bioactive material as claimed in Claim 11 wherein the molecules of the oligosaccharide component are predominantly of a molecular species:



in which

X is ΔHexA(±2S)-GlcNSO$_3$(±6S),

Y is IdoA(±2S)-GlcNSO$_3$(±6S),

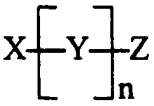
Z is IdoA-GlcR(±6S) or

IdoA(2S)-GlcR(±6S)

where R is NSO$_3$ or NAc, and

n is in the range of 3 to 7.

13. Bioactive material as claimed in Claim 11 wherein the molecules of the oligosaccharide component are composed predominantly of a molecular species:



in which

X is ΔHexA(±2S)-GlcNSO$_3$(±6S),

Y is IdoA(±2S)-GlcNSO$_3$(±6S),

Z is IdoA-GlcR(±6S) or

IdoA(2S)-GlcR(±6S)

where R is NSO$_3$ or NAc, and

n is less than 3.

14. Bioactive material as claimed in any of the preceding claims wherein the molecules of the oligosaccharide component also carry a molecule of a drug or other therapeutically active agent linked thereto.

15. Bioactive material as claimed in Claim 14 wherein the molecules of the drug or other therapeutically active agent are attached directly to the reducing end of the oligosaccharide chains.

16. Bioactive material as claimed in Claim 14 wherein the molecules of the drug or other therapeutically active agent are attached at the reducing end of the oligosaccharide chains through a spacer arm or linkage.

17. Bioactive material as claimed in Claim 14, 15 or 16 wherein the drug is in the form of a water-soluble prodrug.

18. Bioactive material as claimed in any of the preceding claims wherein the oligosaccharide component promotes or stimulates biological activity of the growth factor component.

19. Bioactive material as claimed in any of Claims 1 to 17 wherein the oligosaccharide component inhibits biological activity of the growth factor component.

20. Bioactive material as claimed in Claim 19 wherein the molecules of the oligosaccharide component are less than a decasaccharide in length.

21. A method of preparing in a one step procedure the bioactive material claimed in any of the preceding claims, said method comprising treating a preparation of heparan sulphate (HS) oligosaccharide with cross-linking reagents to form a succinimide ester derivative in the presence of said growth factor, followed by a purification stage.

22. A method of preparing the bioactive material claimed in any of Claims 1 to 20 comprising treating a preparation of heparan sulphate (HS) oligosaccharide with cross-linking reagents to form a succinimide ester

derivative in a first step, and then reacting said succinimide ester activated derivative of the oligosaccharide with a preparation of said growth factor in a second step.

23. A method of preparing the bioactive material claimed in any of Claims 1 to 20 comprising mixing a preparation of the growth factor component with a photoreactive heparan sulphate oligosaccharide and illuminating with light of appropriate wavelength to activate said photoreactive oligosaccharide.

24. A method of preparing the bioactive material claimed in any one of Claims 14 to 17 wherein the drug or other therapeutically active agent is coupled to the oligosaccharide component in a first stage and the conjugate compound so formed is then covalently cross-linked to the growth factor in a second stage.

25. A bioactive conjugate compound composed of molecules of a heparin or heparan sulphate oligosaccharide attached at their reducing ends, either directly or through a spacer arm or linkage, to a drug (or prodrug) molecule.

26. A pharmaceutical formulation for therapeutic use in modulating the activity of a heparin-binding growth factor in the course of therapeutic treatment of a mammal, said formulation comprising a composition of bioactive material as claimed in any of Claims 1 to 20 in admixture with a pharmaceutically acceptable carrier therefor.

27. A pharmaceutical formulation as claimed in Claim 26 in unit dosage form for administering to a mammal, said bioactive material also carrying molecules of a drug or other therapeutic agent.

28. A pharmaceutical formulation for delivery of a drug or other therapeutic agent to a mammal in need of treatment, said formulation comprising a therapeutically effective amount of bioactive material as claimed in any of Claims 14 to 17 together with a pharmaceutically acceptable carrier therefor.

29. A pharmaceutical formulation as claimed in any of Claims 26 to 28 suitable for oral, rectal, topical, spinal, intramuscular, intravenous or other kind of parenteral administration.

30. Use of bioactive material as claimed in any of Claims 1 to 20 for the manufacture of a medical preparation for administering to a mammal to modulate growth factor activity and/or for carrying out targetted drug delivery in the course of therapeutic treatment.

31. A method of manufacturing a medical preparation for administering to a mammal to modulate growth factor activity, said method comprising mixing an effective growth factor activity modulating amount of a bioactive material as claimed in any of Claims 1 to 20 with a compatible pharmaceutically acceptable additive, carrier, diluent or excipient.

32. A method of manufacturing a medical preparation for administering to a mammal for carrying out targetted drug delivery in the course of therapeutic treatment, said method comprising mixing with a compatible pharmaceutically acceptable additive, carrier, diluent or excipient a bioactive material as claimed in any of Claims 1 to 20 which contains a therapeutically effective amount of a drug (or prodrug) linked to the oligosaccharide component.

33. A method as claimed in Claim 31 or in any of Claims 21 to 23, wherein the oligosaccharide component has the effect in use of stimulating or enhancing the biological activity of the growth factor component.

34. A method as claimed in Claim 31 or in any of Claims 21 to 23, wherein the oligosaccharide component has the effect in use of inhibiting the biological activity of the growth factor component.

35. A method of modifying a heparin-binding growth factor to improve its suitability for administration to a mammal in the course of therapy, said method comprising coupling said growth factor to a heparin or heparan sulphate oligosaccharide to form a covalently cross-linked conjugate.

36. A method of modifying a drug or prodrug to facilitate administration to a mammal and targetted delivery to cells having specific growth factor receptors, said method comprising coupling molecules of said drug or prodrug to molecules of a heparin or heparan sulphate oligosaccharide which in turn are covalently coupled or cross-linked to molecules of the specific growth factor that interacts with said receptors.

37. Bioactive material as claimed in Claim 18, insofar as it depends from Claim 8 or 9, for therapeutic use as an active FGF-activity stimulating agent for promoting healing or tissue repair in treating mammals in need of such treatment in connection with wound healing, bone healing, nerve regeneration, duodenal or venous ulcers, various ocular and retinal disorders, ischaemia, or other conditions requiring tissue repair, or for protecting tissues against serious damage during radiation treatment.

38. Bioactive material as claimed in Claim 19 or 20, when depending from Claim 8 or 9, for therapeutic use as an active FGF-activity inhibiting agent for controlling or reducing cell growth or proliferation in treating mammals in need of such treatment in connection with diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g. Herpes Simplex type 1), restenosis following angioplasty, or other conditions where there is a requirement to inhibit FGF growth factor activity.

39. A pharmaceutical formulation or composition for medical use comprising a therapeutically effective non-toxic amount of an FGF-activity modulating agent comprising bioactive material as claimed in Claims 8 or 9, together with a pharmaceutically acceptable carrier or vehicle.

40. Use of a bioactive material as claimed in any of Claims 1 to 20, for the manufacture of a medical preparation either for use as a growth factor inhibitor

in the treatment of diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g. Herpes Simplex type 1), or restenosis following angioplasty or for use in stimulating growth factor activity for promoting repair of damaged tissues in conditions such as wound healing, bone healing, nerve regeneration, duodenal ulcers, various ocular and retinal disorders, atherosclerosis, degenerative muscle disorders, ischaemia, or for protecting tissues against serious damage during radiation treatment.

41. A pharmaceutical composition or formulation for use in controlling the activity of fibroblast growth factors in mammals either for promoting tissue repair or for inhibiting cell growth or proliferation in the treatment of disorders resulting therefrom, said composition or formulation comprising a therapeutically effective amount of bioactive material as claimed in any of Claims 1 to 20.

42. A method of treating a mammal to promote healing or tissue repair in the case of wounds, duodenal or venous ulcers, various ocular and retinal disorders, atherosclerosis, degenerative muscle disorders, or ischaemia, or for promoting bone healing or nerve regeneration, or for protecting tissues against serious damage during radiation treatment, said method comprising administering to the mammal in need of such treatment an effective amount of bioactive material as claimed in Claim 18.

43. A method of therapeutic treatment of a tumour or other cell proliferation disorder carried out on a mammal, said method comprising administering to said mammal an effective growth factor activity inhibiting amount of a bioactive material as claimed in Claim 19 or 20.

44. A method of treating diabetic retinopathy, capsular opacification, proliferative vitreoretinopathy, tumour angiogenesis, cancer cell growth and

metastasis, rheumatoid arthritis, mild muscular dystrophy, Alzheimer disease, various viral infections (e.g. Herpes Simplex type 1), or restenosis following angioplasty in mammals by inhibiting FGF growth factor activity, which method comprises administering to a mammal in need of such treatment an effective amount of a bioactive material as claimed in Claim 19 or 20.

FIG. 1

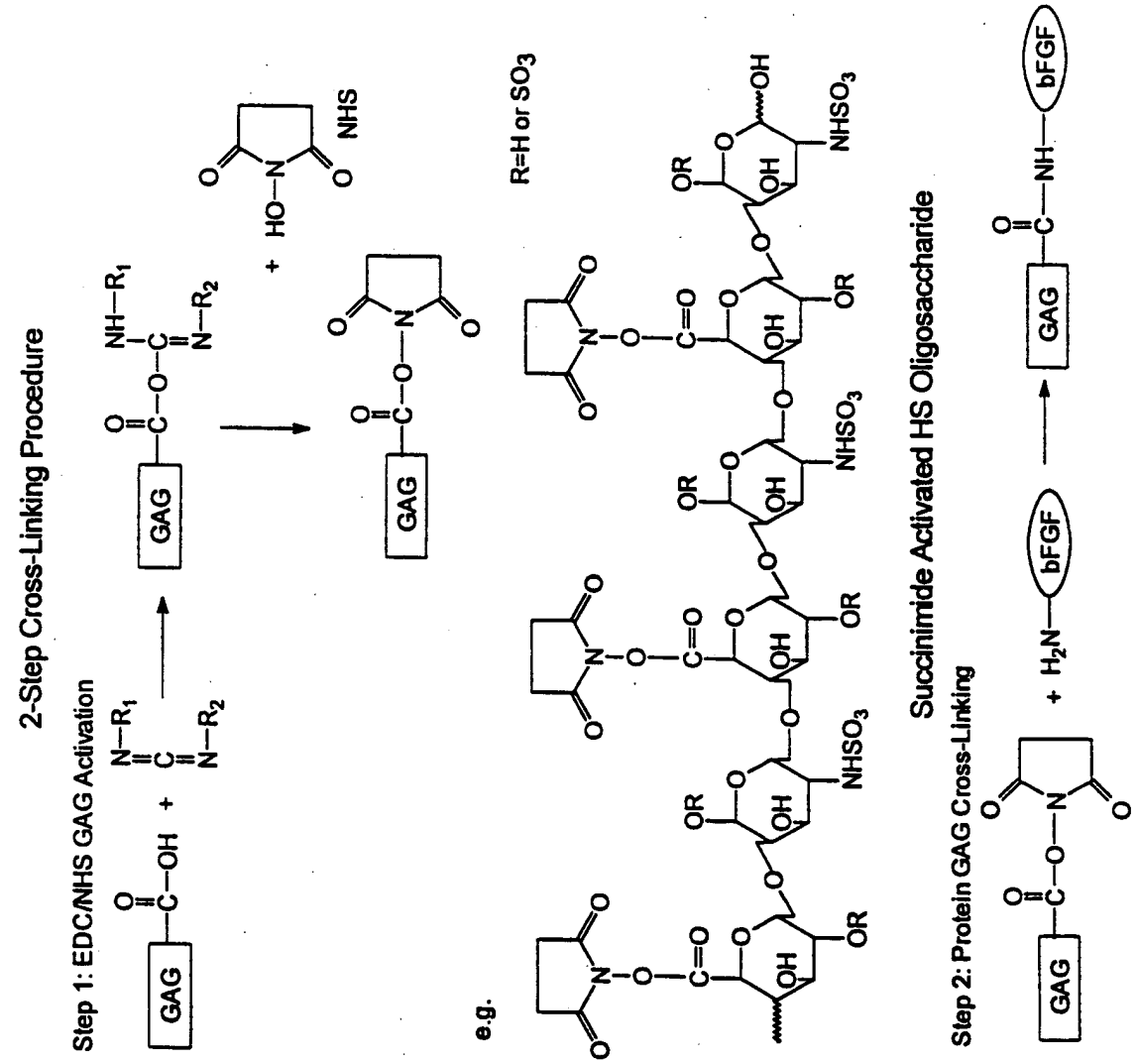

2-Step Cross-Linking Procedure
Step 1: EDC/NHS GAG Activation
GAG
NHS
R=H or SO3
e.g.
Succinimide Activated HS Oligosaccharide
Step 2: Protein GAG Cross-Linking
bFGF

FIG. 2A

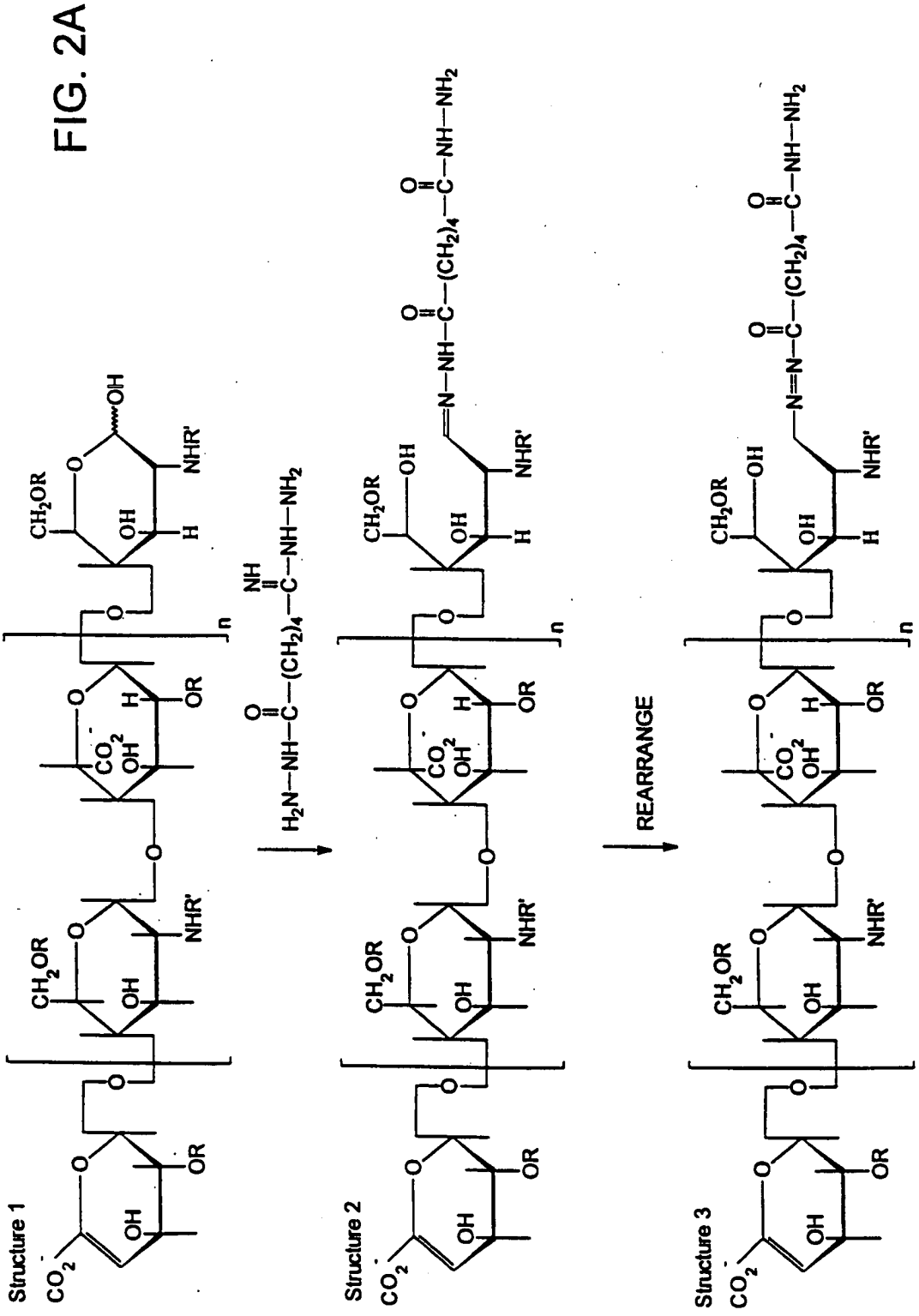


FIG. 2B

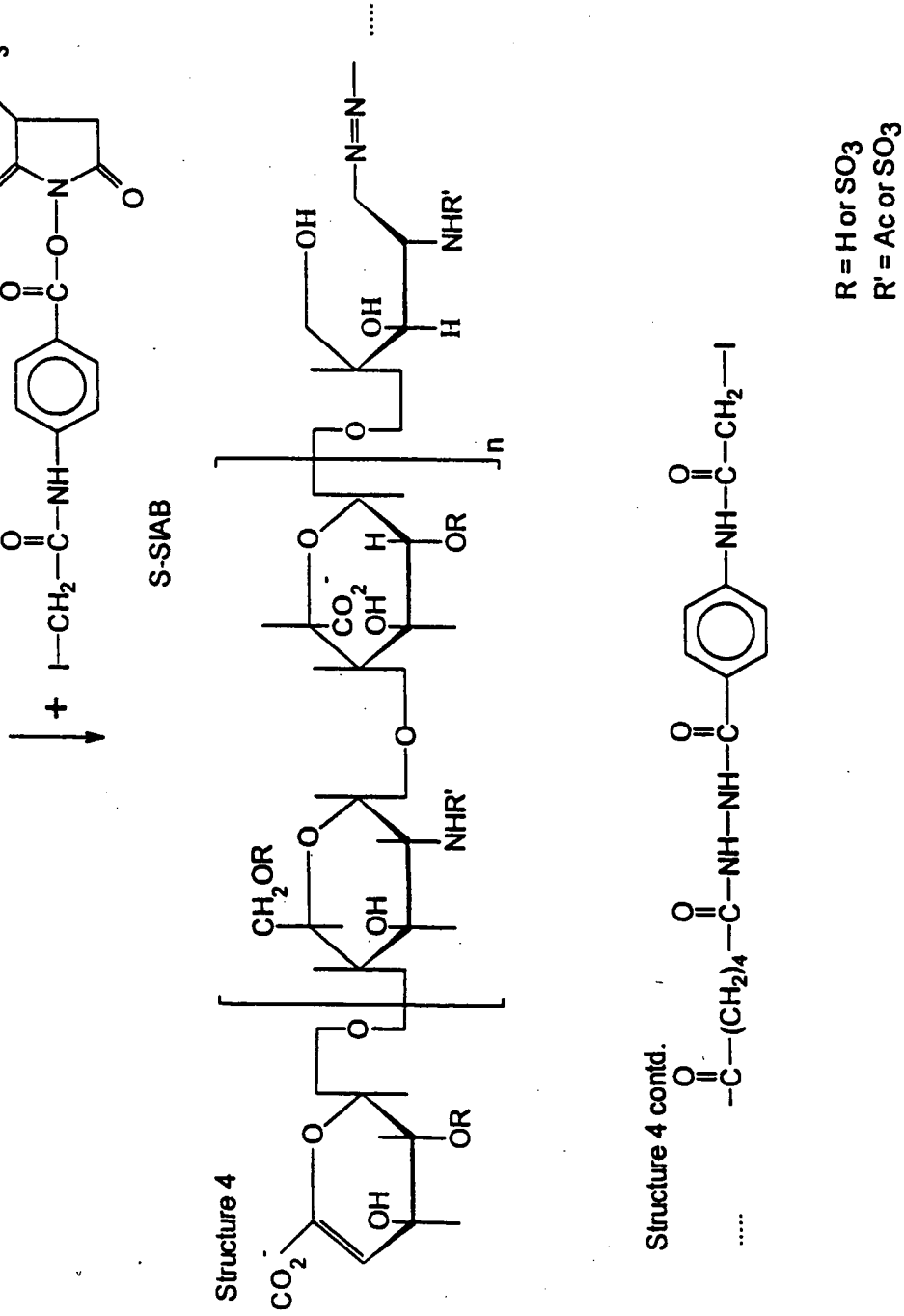


FIG. 3A

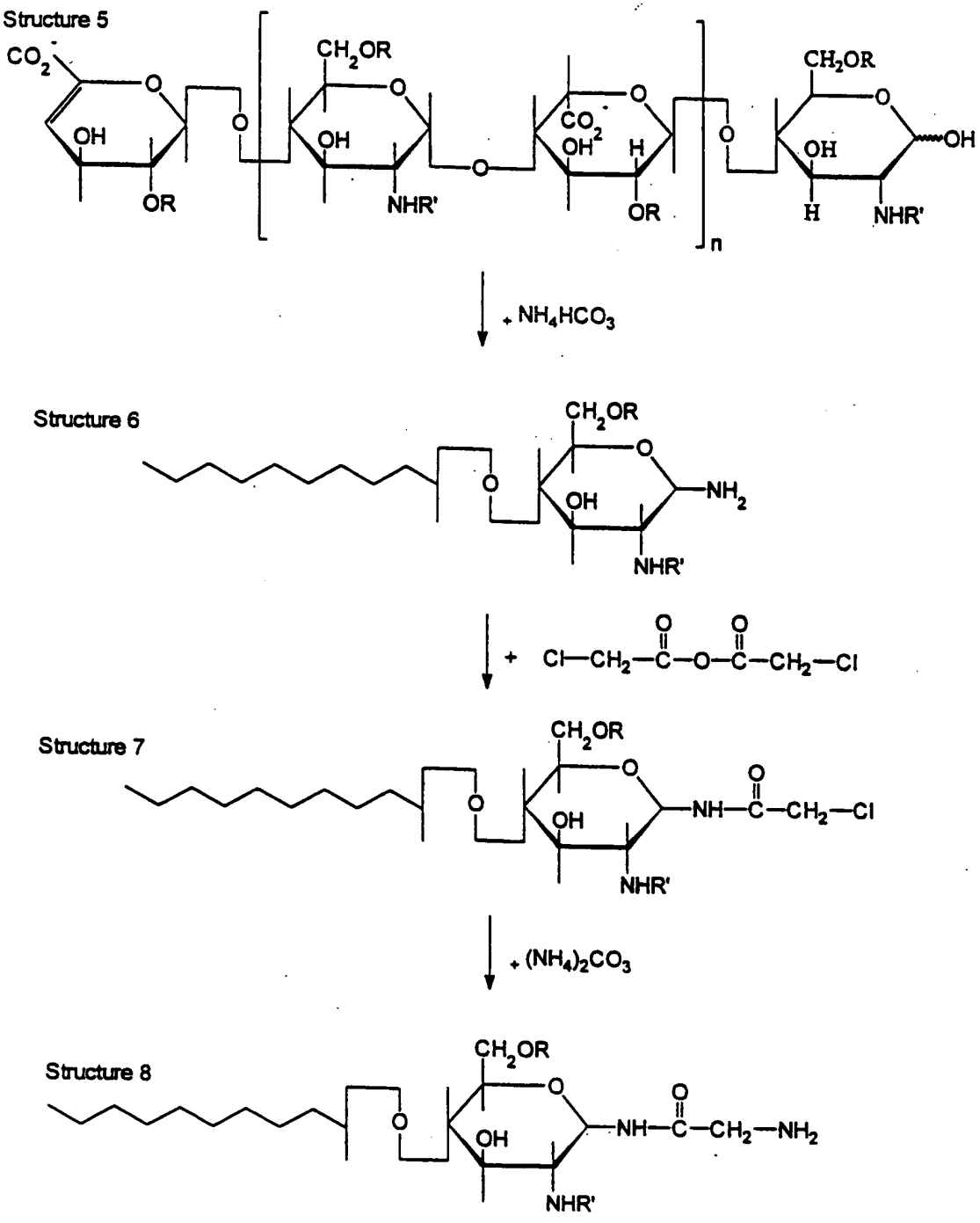

Structure 5
+ $NH_4HCO_3$
Structure 6
Structure 7
+ $(NH_4)_2CO_3$
Structure 8

FIG. 3B

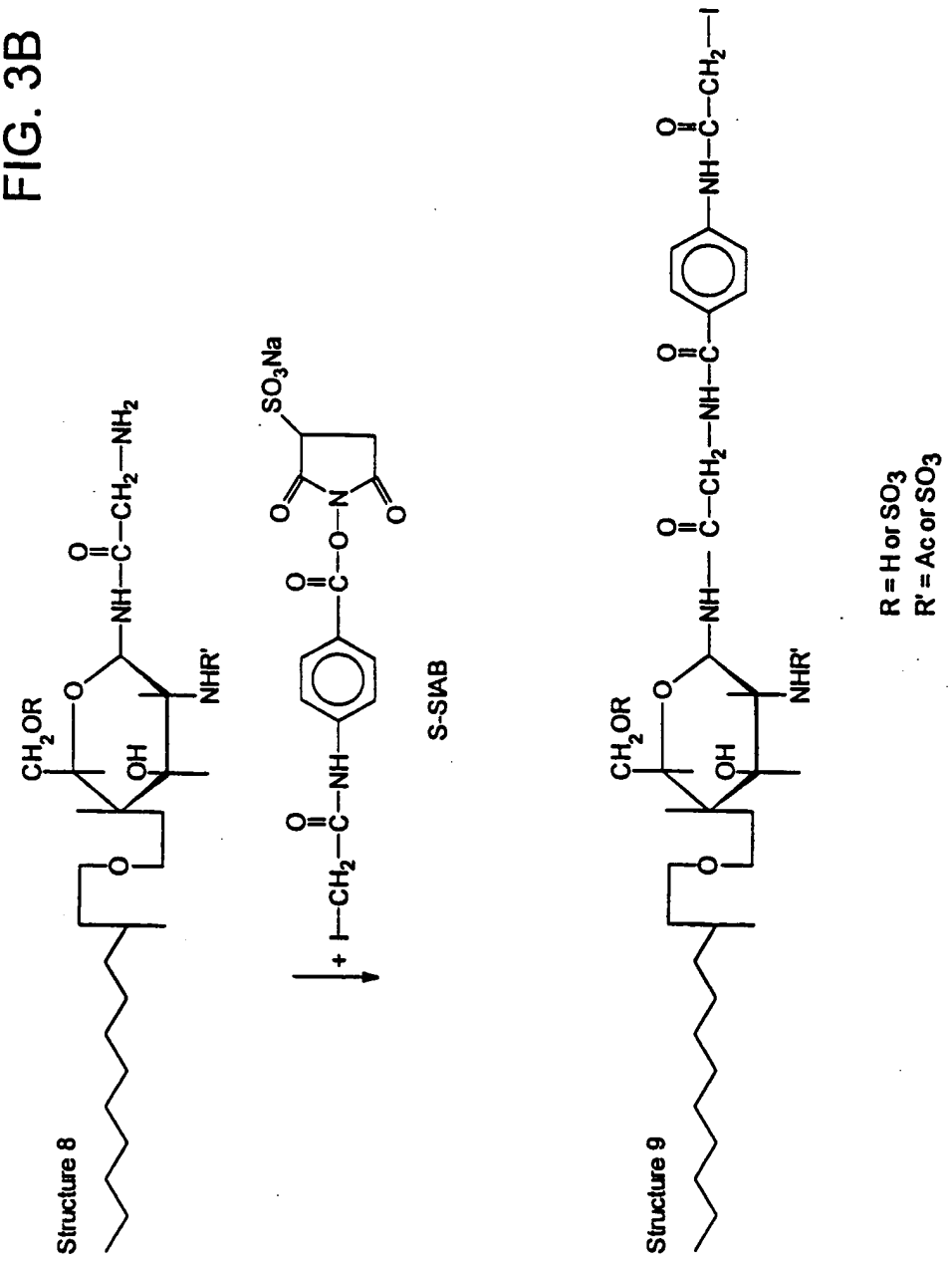
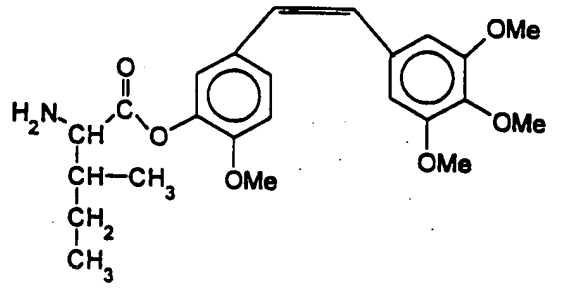
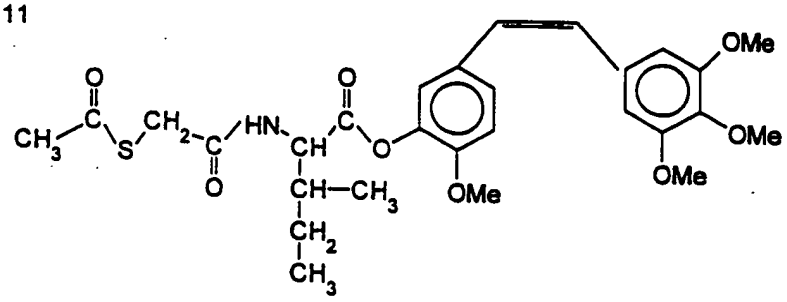


FIG. 4

Structure 10




Structure 11




Structure 12

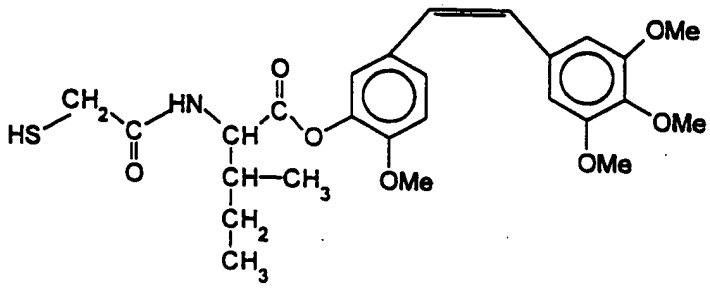


FIG. 5A

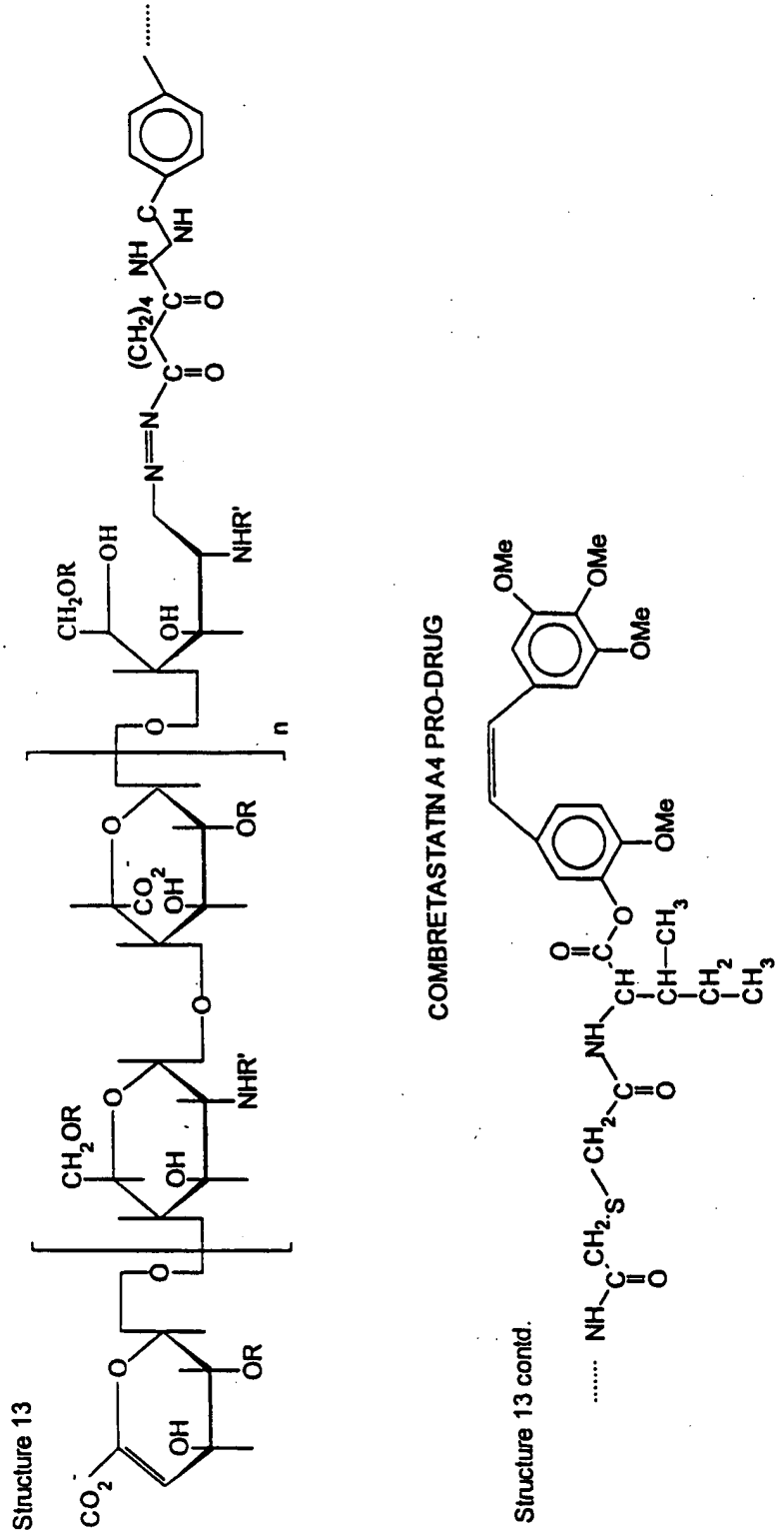

SPACER ARM
HS OLIGOSACCHARIDE
Structure 13
COMBRETASTATIN A4 PRO-DRUG
Structure 13 contd.

FIG. 5B

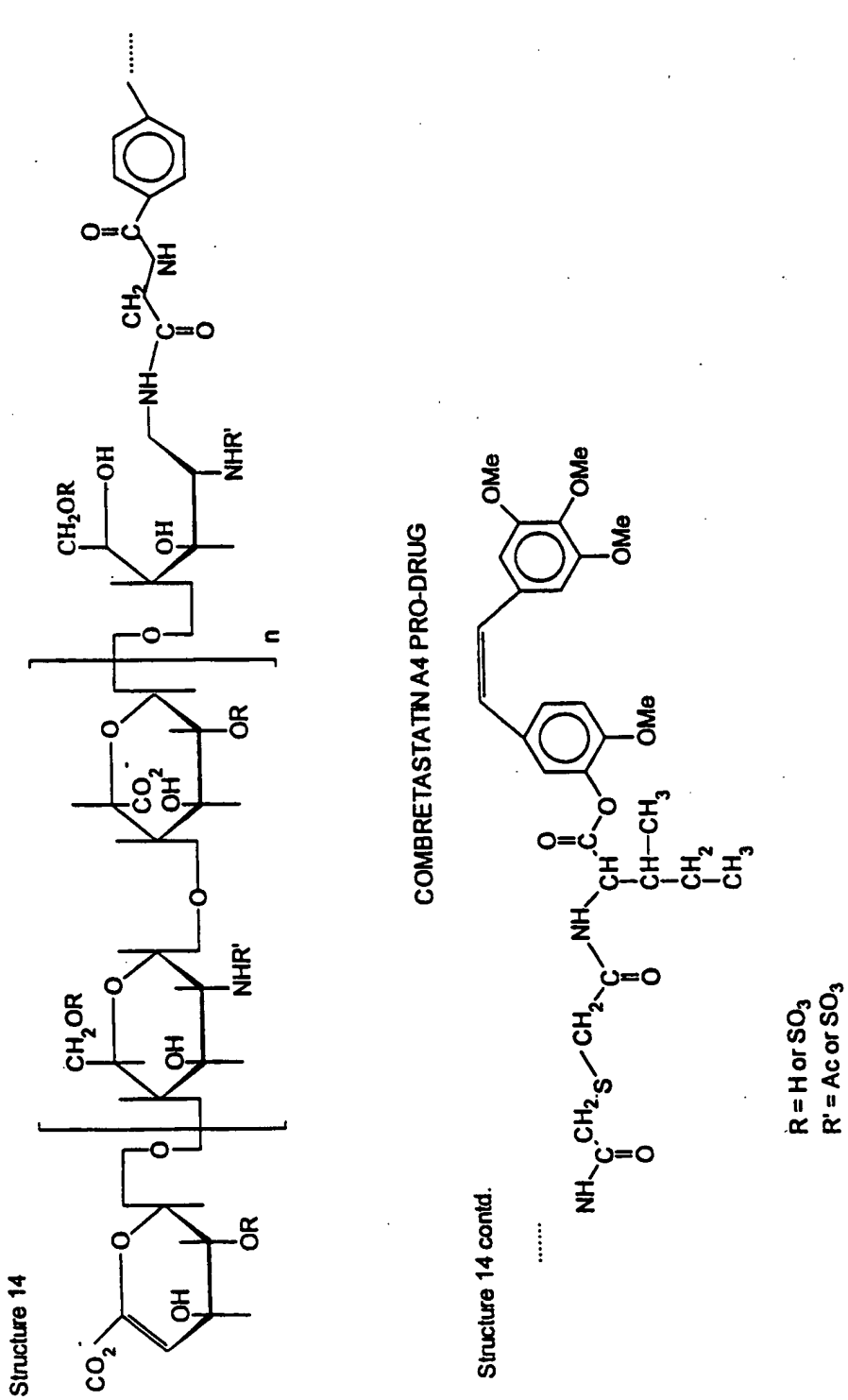
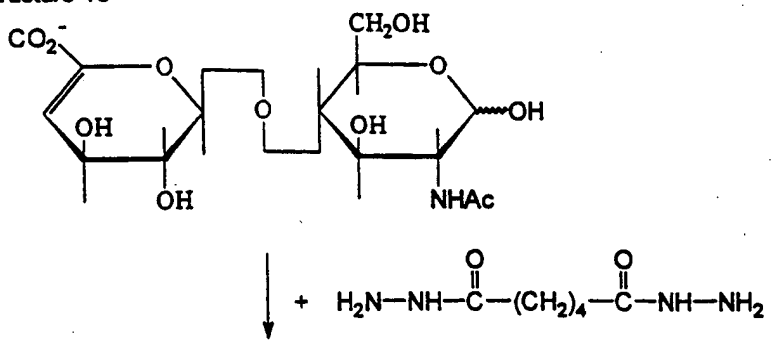
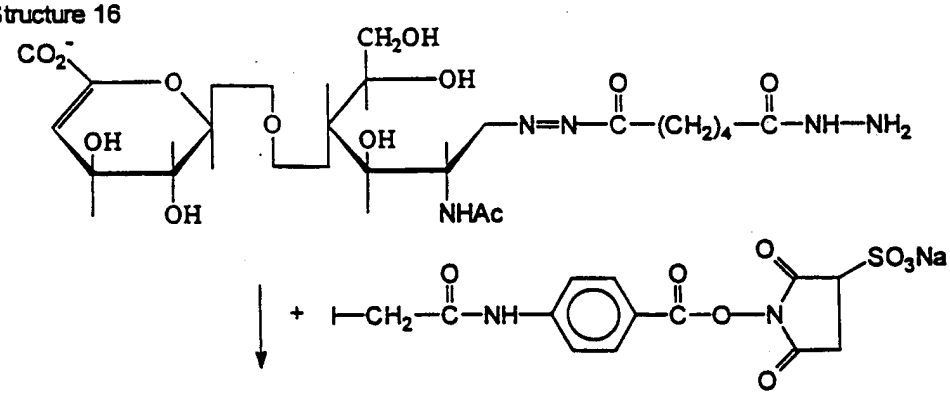

SPACER ARM
HS OLIGOSACCHARIDE
Structure 14
COMBRETASTATIN A4 PRO-DRUG
Structure 14 contd.
R = H or SO3
R' = Ac or SO3

FIG. 6

Structure 15



Structure 16



Structure 17

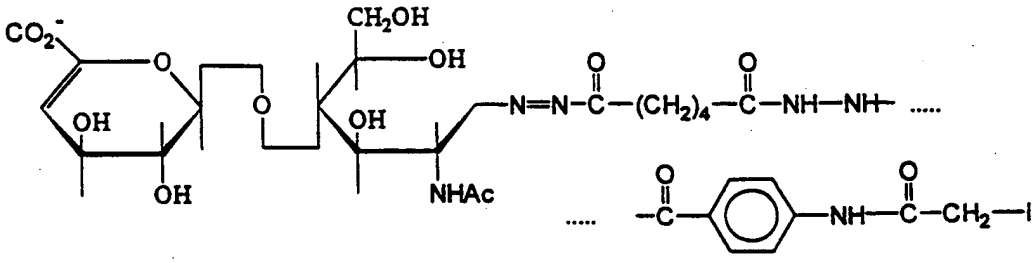


FIG. 7

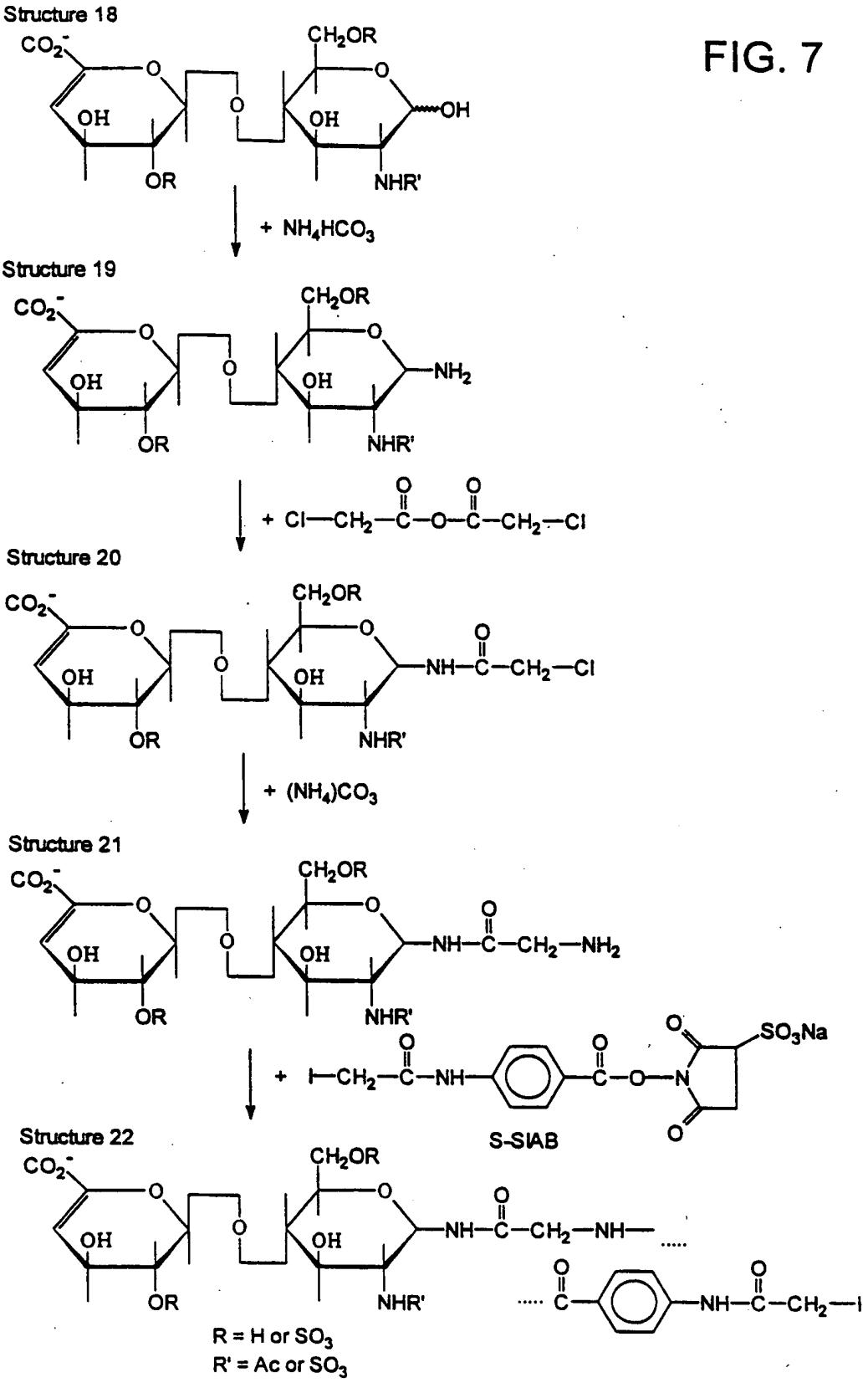

Structure 18
+ $NH_4HCO_3$
Structure 19
+ Cl—$CH_2$—C(=O)—O—C(=O)—$CH_2$—Cl
Structure 20
+ $(NH_4)CO_3$
Structure 21
+ I—$CH_2$—C(=O)—NH— … —$SO_3Na$
S-SIAB
Structure 22
R = H or $SO_3$
R' = Ac or $SO_3$

FIG. 8A

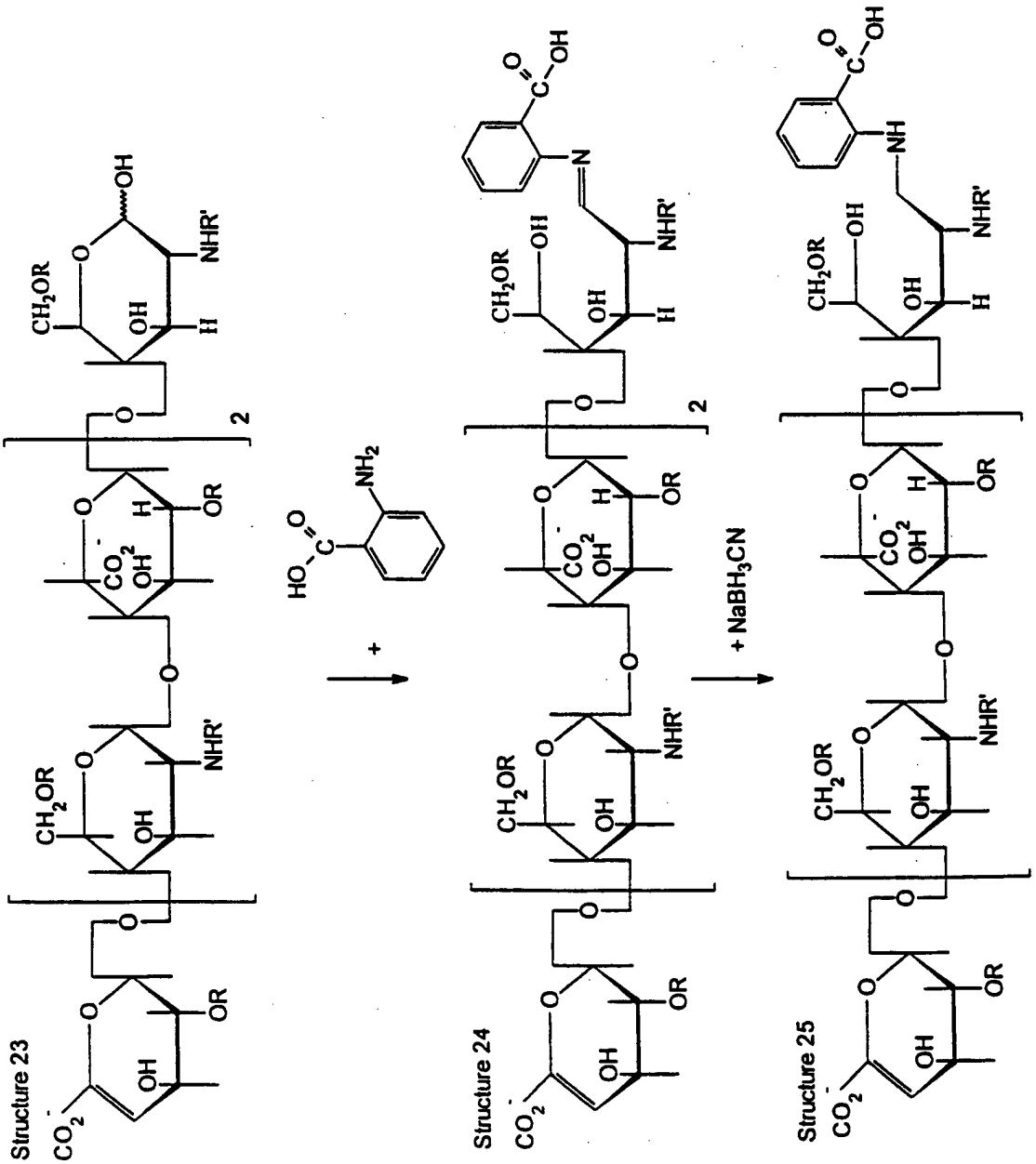


FIG. 8B

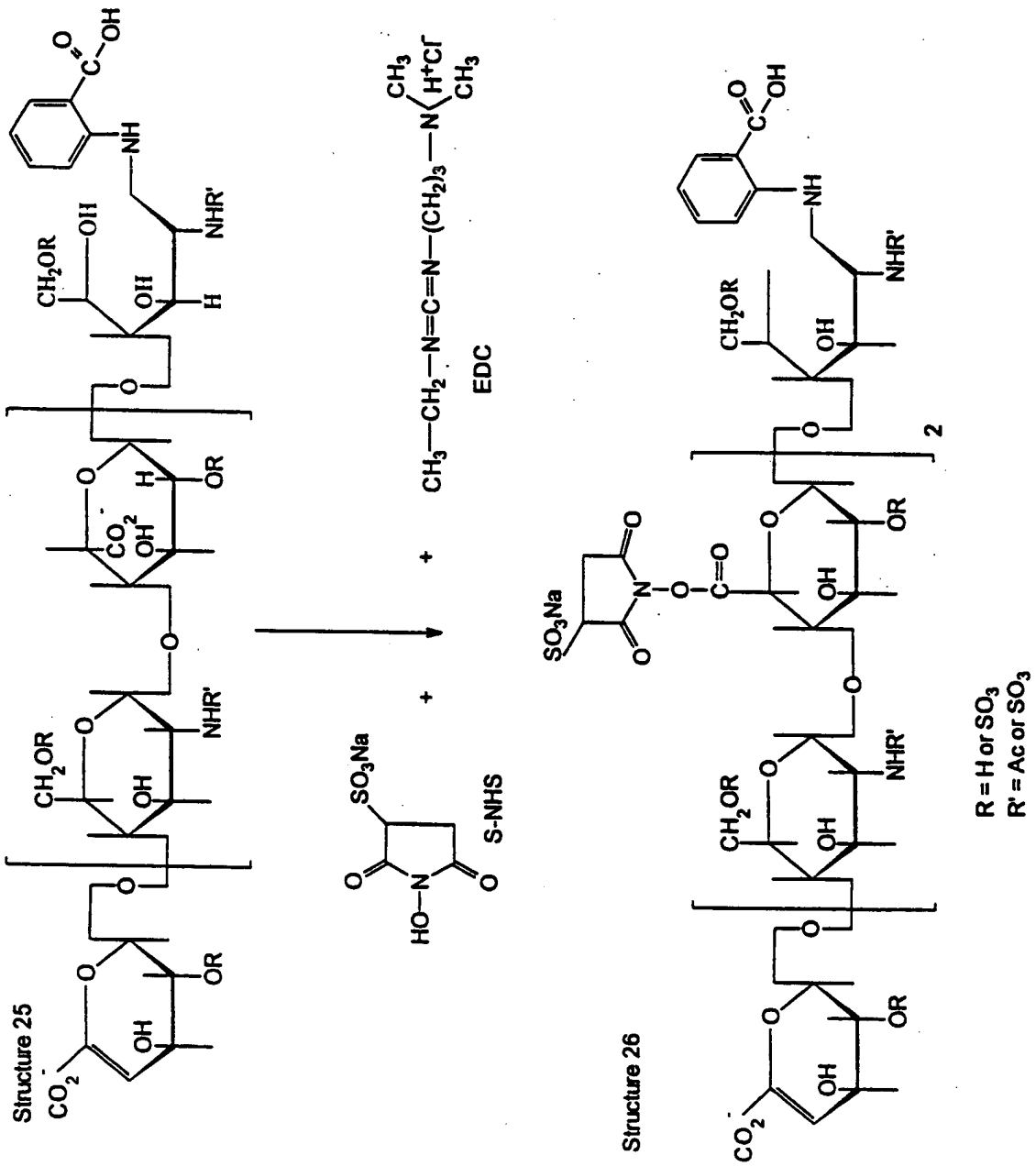


FIG. 9A

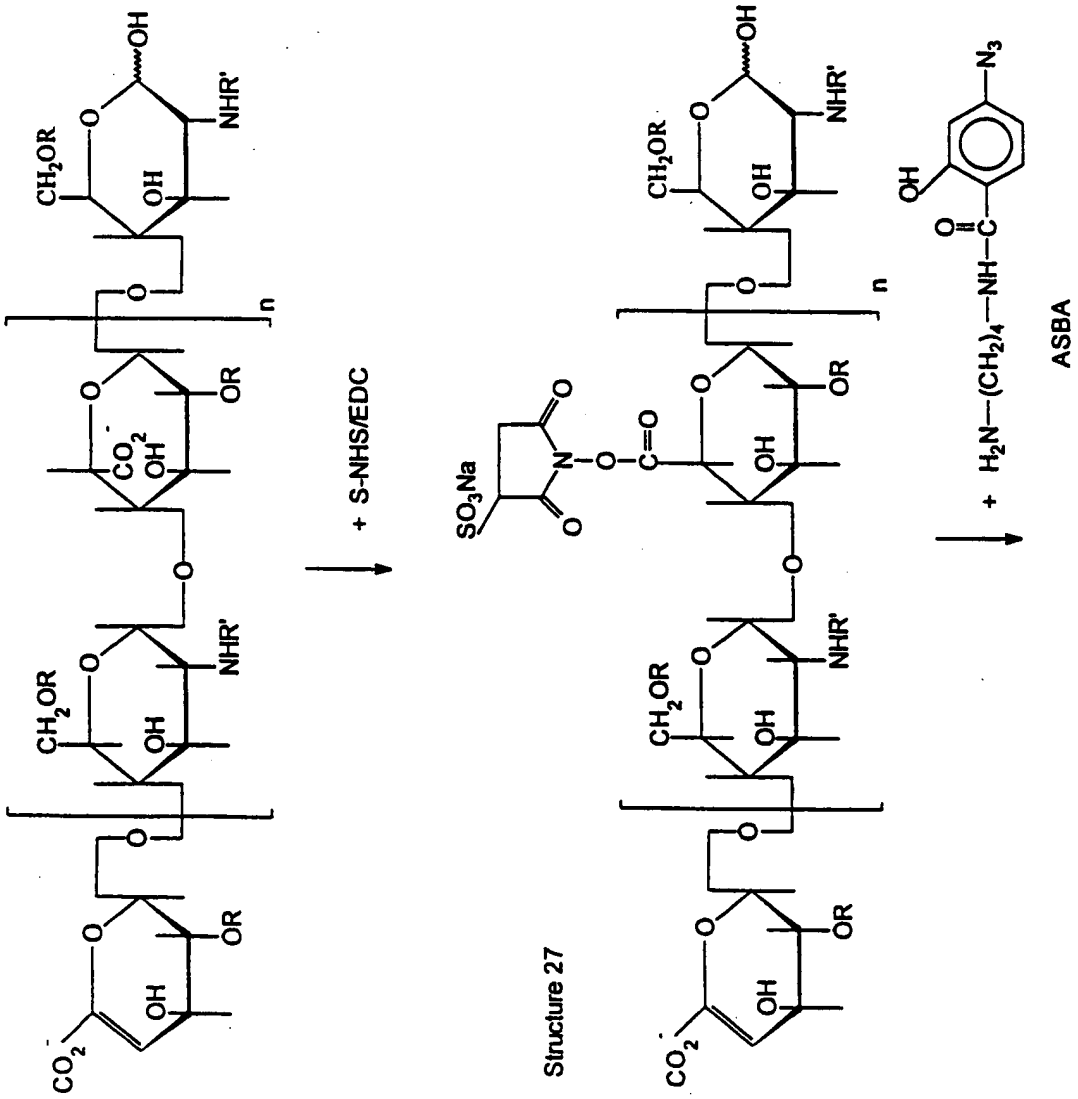


FIG. 9B

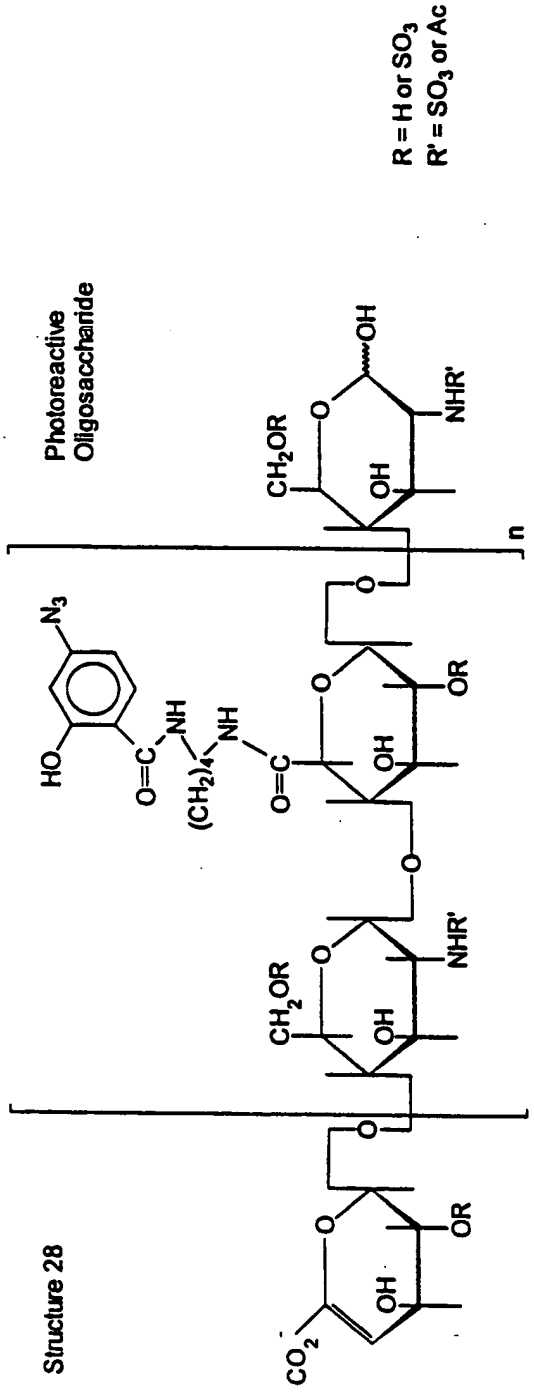
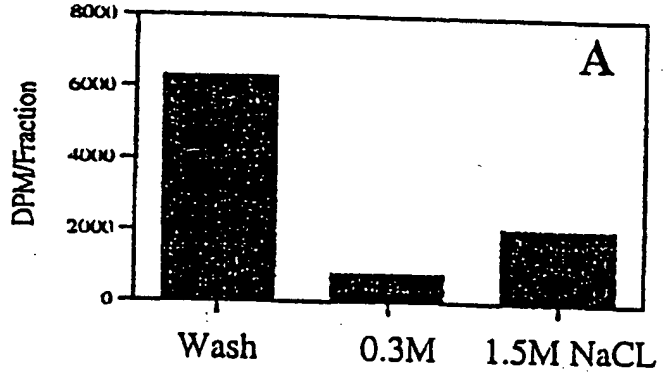

Photoreactive Oligosaccharide
R = H or SO3
R' = SO3 or Ac
Structure 28

Fig. 10


8000
6000
4000
2000
0
DPM/Fraction
A
Wash
0.3M
1.5M NaCL

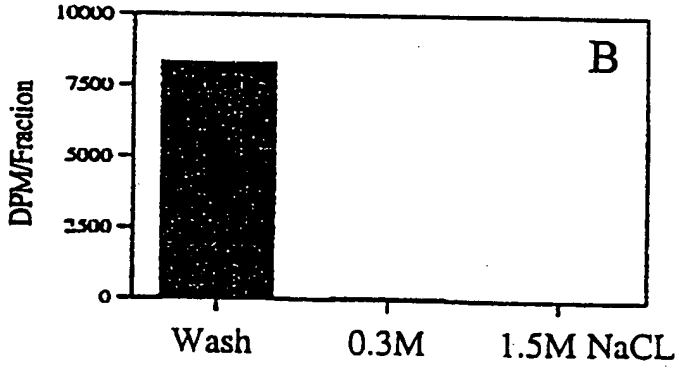

10000
7500
5000
2500
0
DPM/Fraction
B
Wash
0.3M
1.5M NaCL

Fig. 11

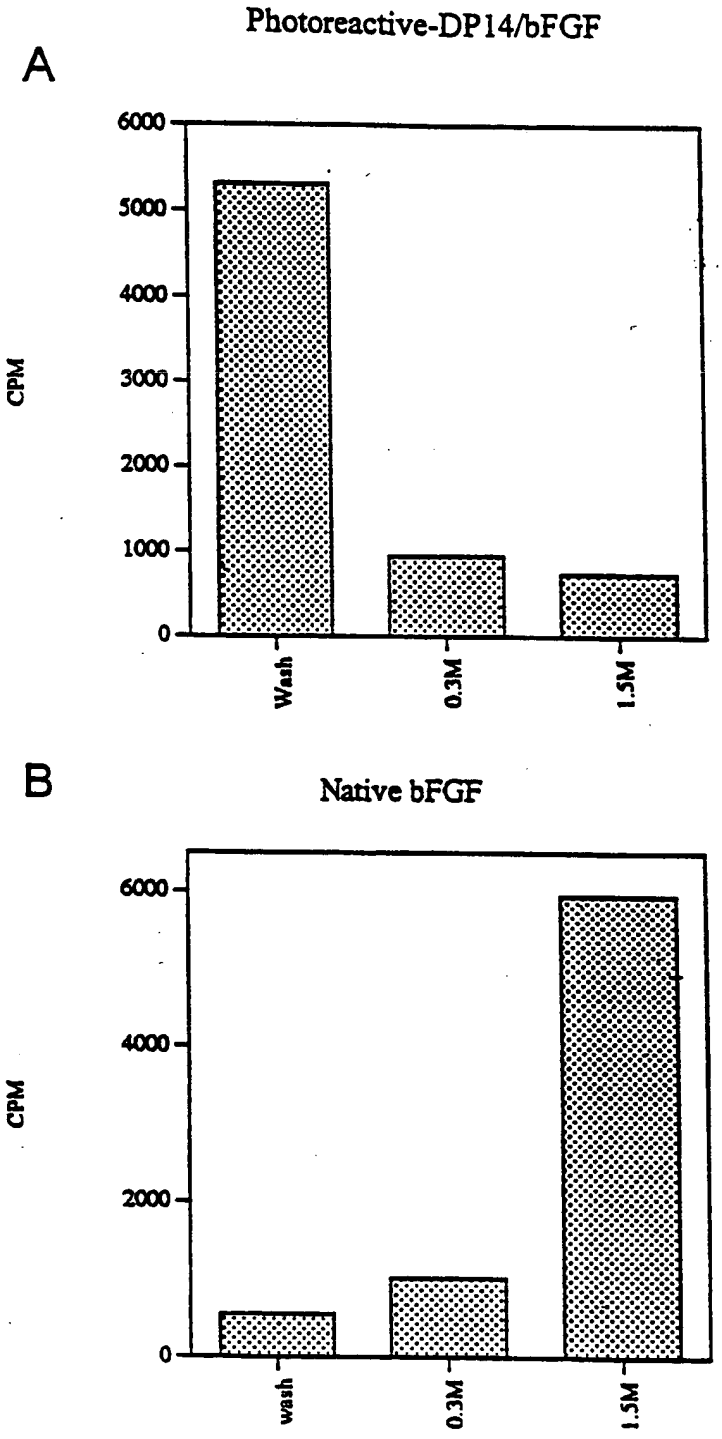

Photoreactive-DP14/bFGF
A
CPM
6000
5000
4000
3000
2000
1000
0
Wash
0.3M
1.5M
B
Native bFGF
CPM
6000
4000
2000
0
wash
0.3M
1.5M

FIG. 12

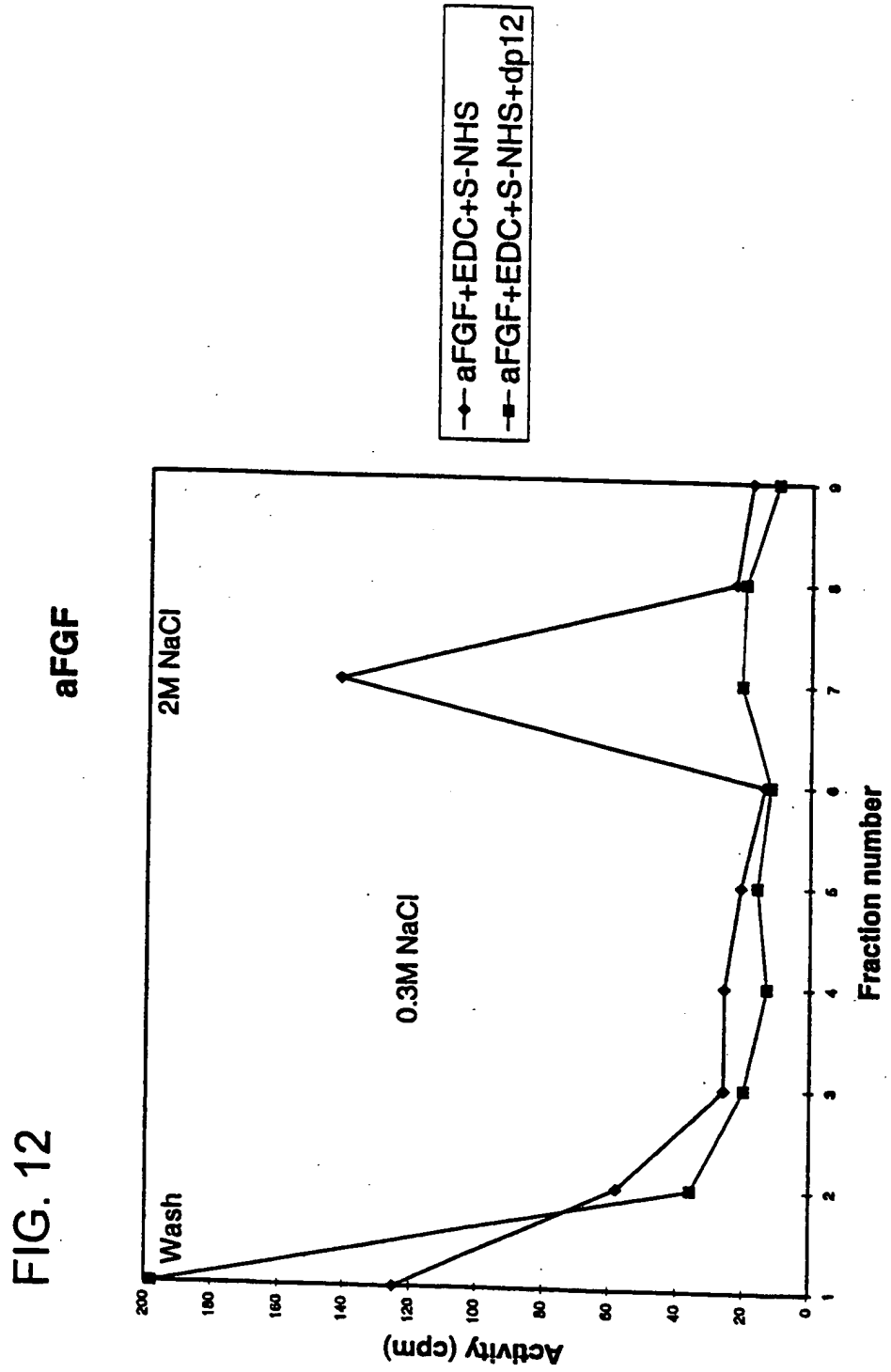

aFGF
aFGF+EDC+S-NHS
aFGF+EDC+S-NHS+dp12
Wash
0.3M NaCl
2M NaCl
Activity (cpm)
Fraction number
0
20
40
60
80
100
120
140
160
180
200
1
2
3
4
5
6
7
8
9

FIG. 13

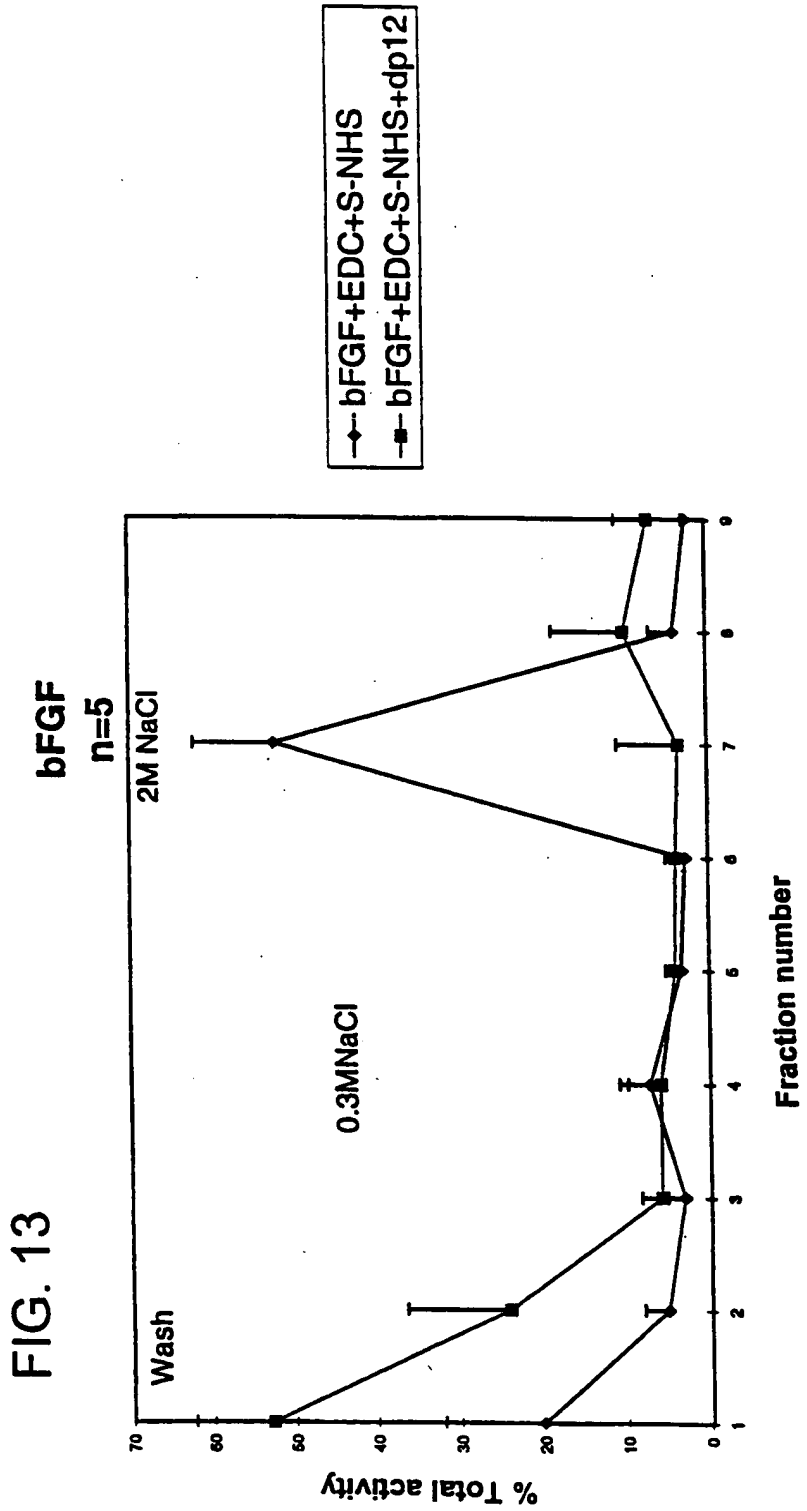

bFGF+EDC+S-NHS
bFGF+EDC+S-NHS+dp12
bFGF
n=5
2M NaCl
Wash
0.3MNaCl
% Total activity
Fraction number
0
10
20
30
40
50
60
70
1
2
3
4
5
6
7
8
9

FIG. 14

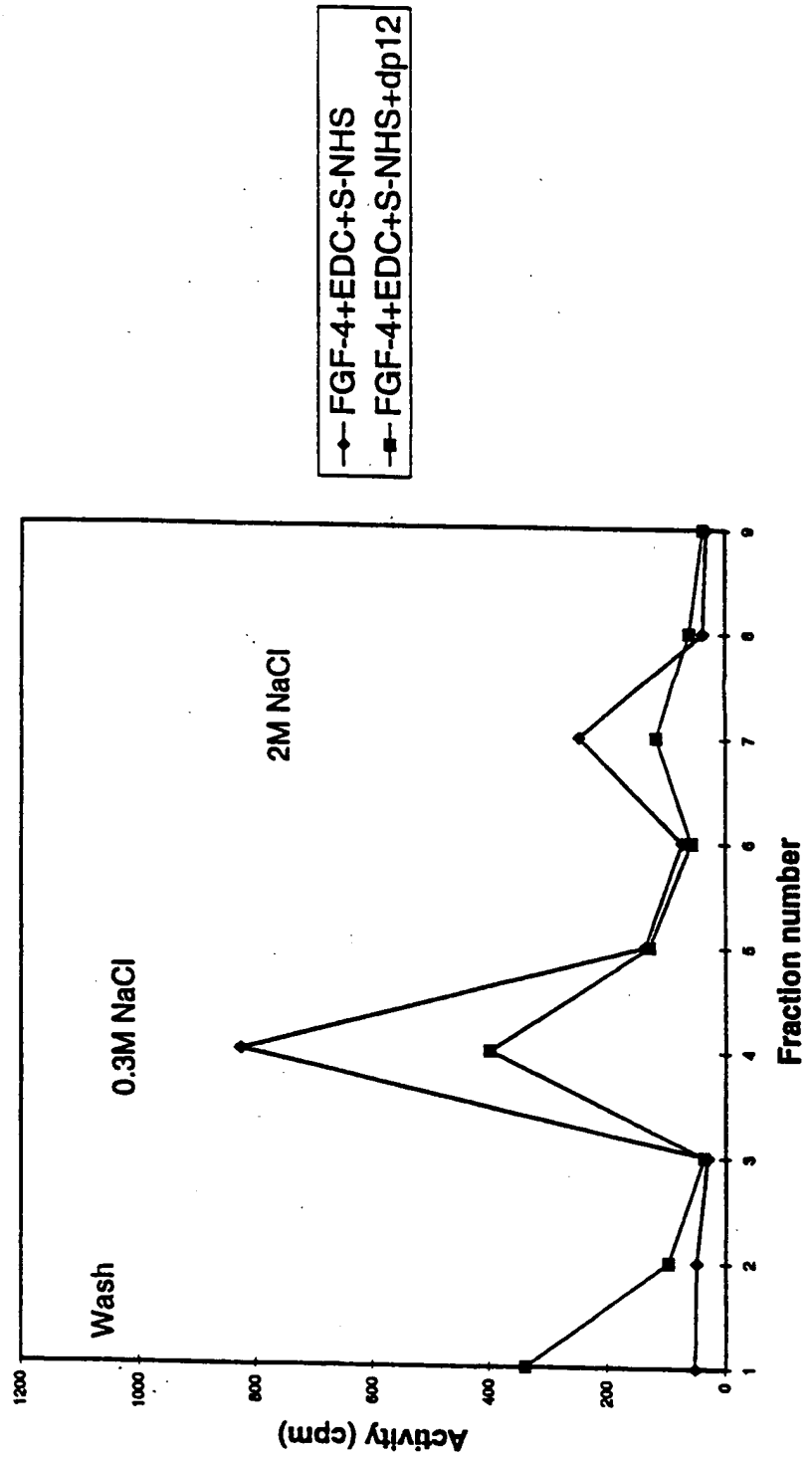

FGF-4
FGF-4+EDC+S-NHS
FGF-4+EDC+S-NHS+dp12
Wash
0.3M NaCl
2M NaCl
Activity (cpm)
Fraction number
0
200
400
600
800
1000
1200
1
2
3
4
5
6
7
8
9

FIG. 15

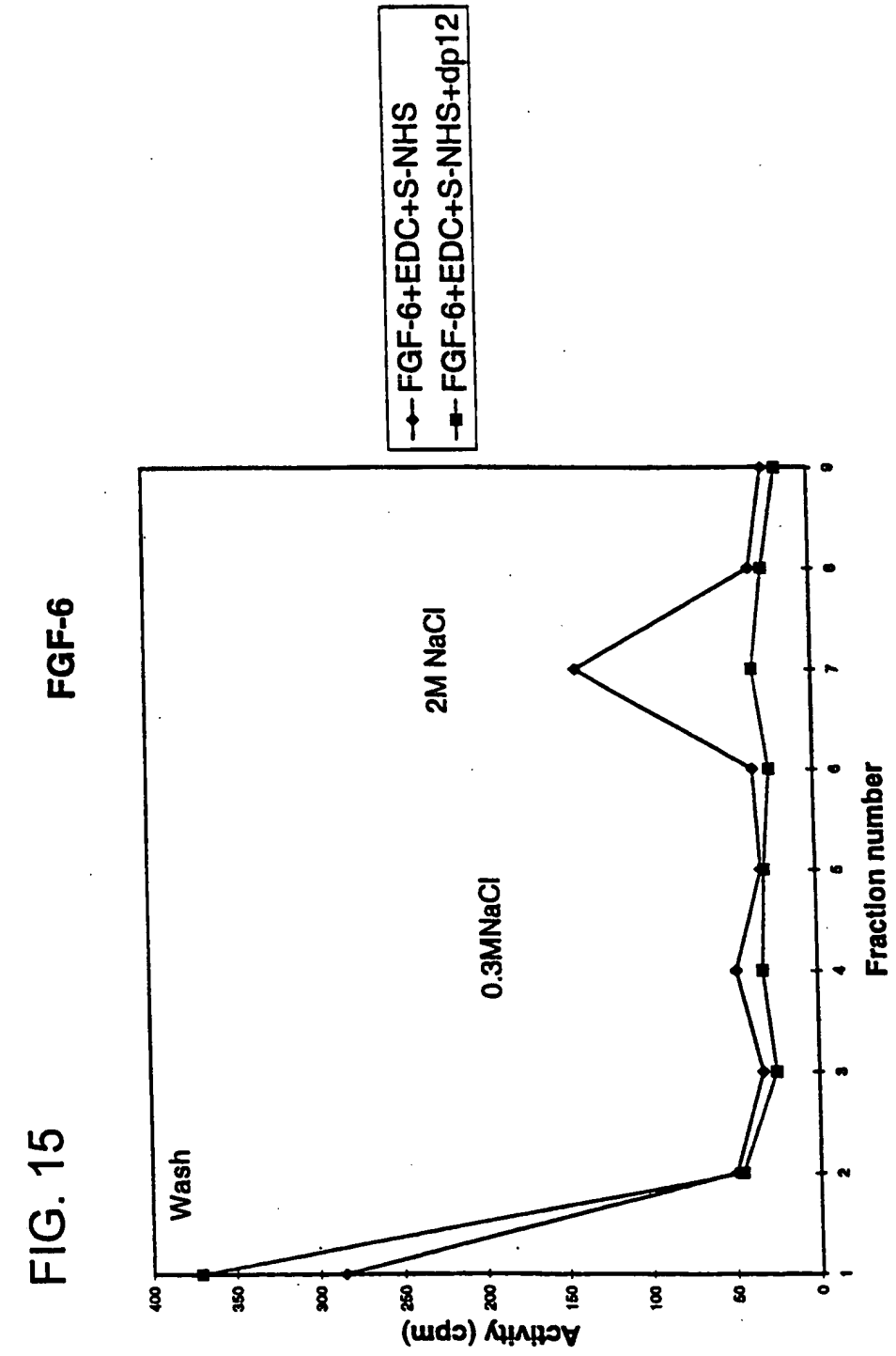
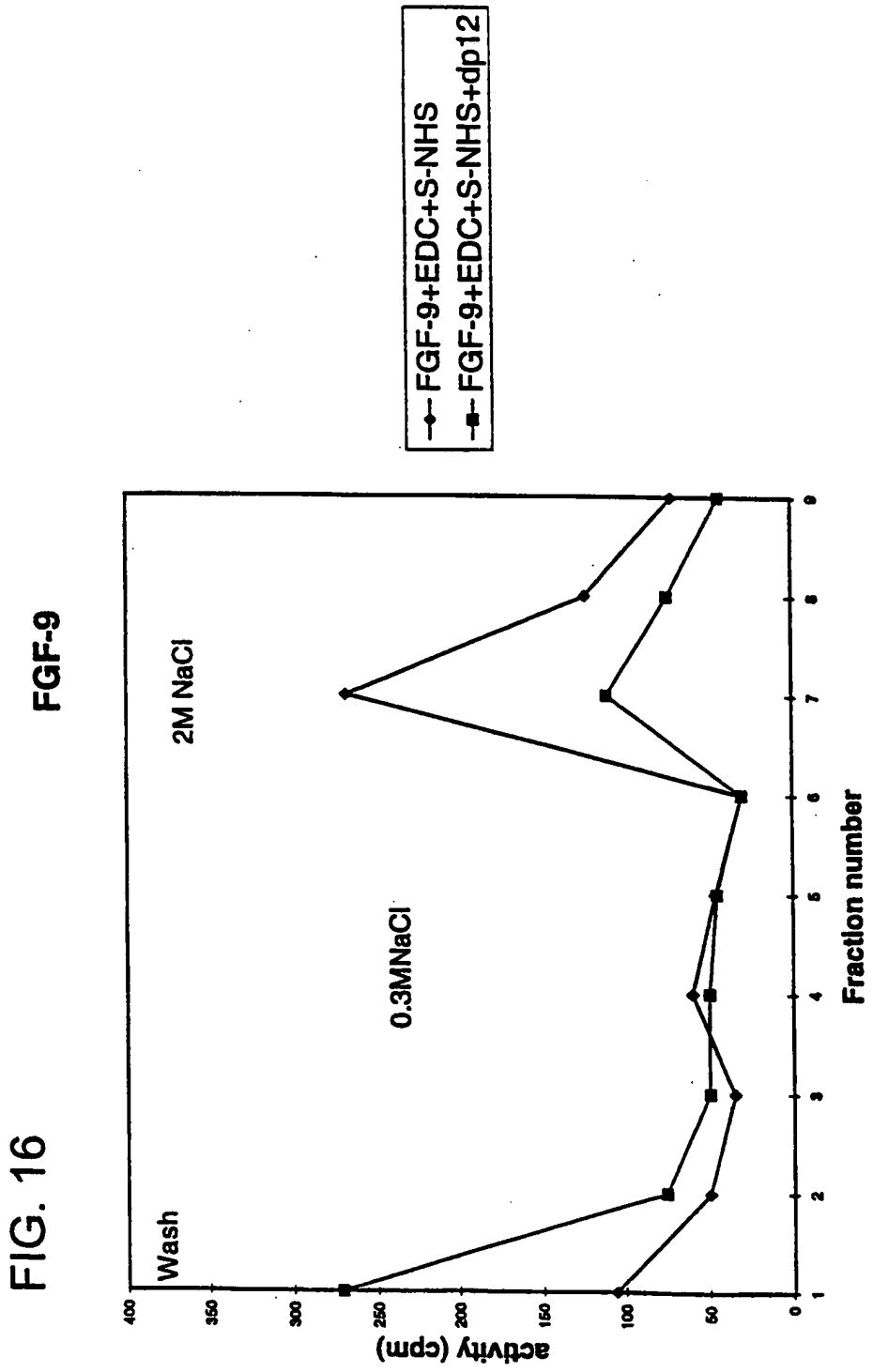

FGF-6
FGF-6+EDC+S-NHS
FGF-6+EDC+S-NHS+dp12
Wash
0.3MNaCl
2M NaCl
Activity (cpm)
Fraction number
400
350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9

FIG. 16


FGF-9
FGF-9+EDC+S-NHS
FGF-9+EDC+S-NHS+dp12
Wash
0.3MNaCl
2M NaCl
activity (cpm)
Fraction number
0
50
100
150
200
250
300
350
400
1
2
3
4
5
6
7
8
9

# INTERNATIONAL SEARCH REPORT

| In tional Application No |
|---|
| PCT/GB 98/03201 |

A. CLASSIFICATION OF SUBJECT MATT
IPC 6 A61K47/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | NADKARNI, V. D. ET AL: "Directional immobilization of heparin onto beaded supports" ANAL. BIOCHEM. (1994), 222(1), 59-67 CODEN: ANBCA2;ISSN: 0003-2697,1994, XP002094365 see abstract see page 61, column 2, paragraph 5; figure 2 --- | 1,14-16 |
| Y | WO 93 19096 A (CANCER RES CAMPAIGN TECH) 30 September 1993 see claims 1,12,20,39,40 --- -/-- | 1-44 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 23 February 1999 | 12/03/1999 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016 | Berte, M |

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# INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/03201

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | HABUCHI, HIROKO ET AL: "Structure of a heparan sulfate oligosaccharide that binds to basic fibroblast growth factor" BIOCHEM. J. (1992), 285(3), 805-13 CODEN: BIJOAK;ISSN: 0306-3275,1992, XP002094366 see abstract | 1-13,18, 35,39 |
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| Y | EP 0 554 898 A (SEIKAGAKU KOGYO CO LTD) 11 August 1993 see page 17, line 36 - line 53; claims 1,2 | 1-44 |

INTERNATIONAL [redacted]RCH REPORT

..ternational application No.
PCT/GB [redacted]3201

**Box I Observation wher certain claims were found unsearchable (Continuati n f it m 1 of first sheet)**

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 31-34,36,42-44
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 31-34,36,42-44 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

**Remark on Protest**

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

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Information on patent family members

International Application No
PCT/GB 98/03201

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Information on patent family members

International Application No
PCT/GB 98/03201

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